Genetic Assessment of Alabama's Redeye Bass (*Micropterus coosae*)

by

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Abstract

The southeastern United States is a hotspot for aquatic biodiversity that is under constant threat of extirpation and extinction due to human disturbance. Molecular markers are relatively new tools that are applicable to conserving these many endemic populations by establishing genetic baselines of hybridizing populations, resolving taxonomic status, identification of genetically pure populations, and helping to better inform management plans for fish species. Of these, SNPs have become preferred due to their utility and reproducibility. Here, I describe the use of a 64 SNP panel diagnostic for the black basses (*Micropterus* spp.) to survey populations of redeye bass (*M. coosae*) in three of their native river systems in the Mobile River Basin: Tallapoosa River, Black Warrior River, and the Cahaba River.

Redeye bass are native to the Mobile River Basin in Alabama and are one of a few black bass species with small geographic ranges. In my first study, I used a diagnostic SNP panel to survey a putative population of redeye bass in the Tennessee River drainage where they are not native. Genetic analyses confirmed the presence of redeye bass outside of their native range in Alabama and served as a baseline for future studies to investigate the presence of these sportfish and to follow these populations over time.

Phenotypic identification is often employed by management agencies to quickly identify individuals in the field, which serves as the basis for population estimates, age and growth estimates, and other factors relating to the management of sportfish. A comparison of phenotypic identification with genetic identification of sympatric Alabama bass and redeye bass revealed substantial error in field identification of the two species and their hybrids. These data should inform management efforts of the ability of genetics to augment future surveys and studies involving these two species.

Redeye bass populations outside of Alabama are designated as species of special concern due to their hybridization with introduced Alabama bass. Little is currently known about the redeye bass populations in Alabama, which is a large portion of their native range. Here, I utilized a diagnostic 64 SNP panel to survey multiple populations of redeye bass across three large river systems in the Mobile River Basin. Hybridization was found to be occurring at very high levels in two of the three river systems and is facilitated by human disturbance. The baseline genetic survey provides a foundation for long-term monitoring of these range-restricted species and should greatly facilitate conservation studies in these unique species.

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Chapter 1 Introduction and Literature Review

1. Southeastern Biodiversity

The freshwater ecosystems of the southeastern United States harbor a diverse array of biodiversity from invertebrates, amphibians, reptiles to fishes (1). The Southeast alone is home to 79% of the freshwater fishes found in the U.S. and Canada (2). Much of this biodiversity is concentrated in the Mobile River Basin (MRB) which is comprised of seven major river systems spread throughout 10 physiographic provinces and encompassing four states (3, 4). Each physiographic province has unique geological, topographical, and other physical characteristics that result in a diverse range of habitats and defines the physical and chemical properties of the waters that flow through them (5). Epochs of time and geological events have contributed to making the Mobile Basin home to hundreds of endemic fauna ranging from mussels, snails, crustaceans, insects, turtles, to fishes (1, 5). The MRB contains over 60 endemic fish species alone (4), and recently described species (6), many of which are lacking basic scientific knowledge and are at risk of imperilment.

Unfortunately, the MRB's record rates of biodiversity are continually affected by record rates of extinctions, with almost 50% of the U.S. extinctions within the last century occurring in this speciose locale (2, 3). The primary causes for this are anthropogenic disturbances such as habitat modification, sedimentation, impoundment, and flow modification (3, 7). The lack of basic life history traits of many of these species coupled with the rate at which large-scale ecosystem modifications have occurred in the MRB makes it very difficult for conservation efforts to keep pace (7). Many of the fishes of the MRB have small geographic ranges making them especially vulnerable to extirpation or extinction (8), while other fish populations have larger ranges that have been fragmented with limited ability for dispersal (9). Additionally, one of the main threats to freshwater biodiversity is the introduction of non-native species that results in changes to the composition of freshwater fishes and can lead to homogenization of native biodiversity (10-12). One taxa where this is extremely common in the black basses (Genus: *Micropterus*).

2. Black Bass

2.1 Biology and Ecology

The black basses, family Centrarchidae, comprise one of the most economically and ecologically important fish taxa in the United States (13, 14). First described in 1802 by French naturalist, Bernard Germain de Lacépède, the Largemouth Bass (*M. salmoides*) and Smallmouth Bass (*M. dolomieu*) encompassed much of the discussion and writing concerning this popular group of sportfish (15, 16). *Micropterus* species are characterized by having an elongate body with a large mouth and an emarginated caudal fin with ctenoid scales (17). Adults spawn annually during the spring, with exact timing dependent upon temperature and geographical location. Males establish a territory, construct a shallow nest, and performs courtship displays to attract a female (18). The male guards the eggs after fertilization and subsequent brood by chasing off predators (17, 18).

Ecologically, black bass are apex predators in a diverse array of freshwater ecosystems and therefore play an important role as indicators of health in aquatic systems (13, 14). The predator-prey dynamic is a crucial component of fisheries ecology as it effects recruitment and other factors that have implications across multiple trophic levels (19). Fishes have a high reproductive capacity and therefore the potential of overgrowth is balanced in part through consumption by piscivorous fish in both natural and impounded habitats, of which the black basses play a key role (20). We now know that there are many more factors at play than just understanding the black bass in a production context. There are a multitude of stochastic properties that can affect population numbers in natural systems such as temperature and water levels (21). Past research has broadened our understanding of ecology to now encompass landscape processes in addition to population dynamics adding to the complexity of understanding and managing this taxa in a broad array of habitats. Due to the ecological importance coupled with the popularity of fishing for black bass, increased attention has been placed on management of these populations, which require an equally diverse range of strategies that has had to evolve over the years.

2.2 Economic Importance and Management

Although they have been introduced around the world (22, 23), black bass are native to the eastern North America (16). The enjoyment of black bass as a sportfishing quarry and as table fare led to major declines in many populations by the early 1900s. The Black bass Act in 1926 dissuaded the commercial harvest and sale of black bass and prevented interstate commerce of this important game fish. The range of black bass has also been increased within the United States by private individuals and state and governmental agencies to establish and enhance sport fisheries in lentic habitats created by impoundments. The adaptability of Largemouth Bass (*Micropterus salmoides*), Smallmouth Bass (*M. dolomieu*), and Spotted Bass (*M. punctulatus*) to both lotic and lentic systems have led to these species inhabiting a broader range of freshwater systems and thereby increasing their popularity as sportfish (14). These translocations have led to deleterious effects on lotic habitats in order to increase production and access to a few of these species well-suited for a wide array of environments (16, 24). These measures contributed to the rise of the black bass as one of the most popular freshwater sportfish in the United States and have contributed large sums of money for conservation to annual revenues of state fish and game agencies charged with managing them (16, 25). As the 20th century came to a close, catch-and-release became more common with an increased focus on managing for size structure rather than harvest limits (26, 27). A more recent management focus has been on conserving the native diversity among the black basses for its value to natural ecosystems (25). Over a century has passed since the work by Dr. James Henshall, a vehement voice of an underappreciated fish, and today the sporting quality of bass and the industry surrounding the pursuit of it must still compete with the mighty salmonid for funding and conservation (28). Although some black bass species are habitat generalists, many black bass species are endemic to small geographic ranges and have evolved to be habitat specialists within those systems. These range-restricted populations of black bass add a lot to the overall diversity within the genus, yet still require a lot of research to determine basic life history characteristics (15, 29).

2.3 Taxonomy

Since the initial description in the early 1800s, the taxonomical status of the black bass remained unchanged for over 100 years until Hubbs and Bailey began to document and describe some relationships among the different species of black bass (30). This seminal work of Hubbs and Bailey resulted in an increase from two to six recognized and accepted black bass species with the additions of the Spotted Bass, Redeye Bass (*M. coosae*), and the Suwanee Bass (*M. notius*) (30). A decade later, there was another revision that formally recognized all black bass taxa as *Micropterus* and described the Florida Largemouth Bass (*M. salmoides floridanus*) as a new subspecies of the northern Largemouth Bass (*M. salmoides*) (31). Originally considered a form of Largemouth Bass from Texas (32), then a subspecies of the Spotted Bass (30), the Guadalupe Bass (*M. treculi*), the State Fish of Texas, was given species status in 1954 (33). Similarly, the Shoal Bass (*M. cataractae*) was originally considered as a form of Redeye Bass (34), before being described as a species at the end of the 20th century (35).

All of the above descriptions and revisions in taxonomy of the black basses were based off of morphological and meristic traits as the structure of DNA had not yet been discovered or had been recently discovered (36). The advent of molecular techniques allowed for a more fine-scale approach to investigating similarities and differences among the black basses (15). There was also an increased desire to understand the distribution, biology, and conservation needs of the rarer black bass species: Guadalupe Bass, Redeye Bass, Suwannee Bass, and the Shoal Bass, which still centered around questions and controversy regarding taxonomic status.

A phylogenetic analysis of mitochondrial DNA (mtDNA) revealed further revision was needed to more accurately reflect the diversity within the black basses. One of the more interesting findings was the genetic distinction within the Largemouth Bass, indicating that Florida Bass warrants species status due to a sequence divergence greater than 3.89% based on mtDNA (37). For reference, the sequence divergence between Smallmouth Bass and Spotted Bass is only 1.20% (38). Additionally, there was a high level of divergence observed between Alabama Bass (*M. henshalli*) and Spotted Bass (15), both formerly believed to be subspecies of Spotted Bass (39). This was not particularly surprising as the two are geographically separated with the Alabama Bass in the clear lakes and rivers of the MRB of Alabama and Georgia, while the Spotted Bass is more broadly distributed in the Ohio and Mississippi River drainages across multiple habitat types (30, 39, 40). These two taxa were shown to be genetically distinguishable with fixed alleles at multiple loci, fixed haplotype differences, and a sequence divergence of

10.28% (15). Investigations into the relationship between sympatric Alabama Bass and Redeye Bass using mtDNA revealed a sister relationship between the two, potentially due to historical hybridization and introgression between these species. Specifically, within the Redeye Bass due to the significant distribution and genetic structuring within that species complex (15). Although this study still left many questions unanswered based on limited sample sizes and sample locations as well as technological limitations, it was the first large-scale molecular investigation into the black bass genus using nuclear, mitochondrial and morphological data.

Building on the molecular work of Kassler et al. 2002, the Alabama Bass was formally described as a species in 2008 using morphological data (41). In addition, the same group investigated variability within the Redeye Bass complex using morphological characteristics and one mitochondrial gene (ND2) based on previous studies supporting unique genetic structuring based on river systems (15), and recognition of Atlantic Slope populations with high levels of divergence from MRB populations (42). In 2013, the Redeye Basses, formerly known collectively as such were split into five different species, each endemic to a certain river system. These were the Coosa, Cahaba, Black Warrior, and Tallapoosa rivers of the MRB and the Chattahoochee River (6). Alabama Bass were left out of these analyses due to their inclusion rendering them paraphyletic, a clear indication that there could be hybrids present in the samples. A follow up study investigating the same relationships using both nuclear and mitochondrial DNA markers verified that the previous study had included hybrids in their species descriptions, an unfortunate possibility when only looking at uniparentally inherited markers that can confound evidence of hybridization (43). While there was a reluctance to accept the validity of the MRB Redeye Bass species descriptions, strong support existed for species recognition of the

Atlantic Slope populations in the Savannah and Altamaha River systems based on reciprocal monophyly and diagnosable genetic traits, and morphological distinctions (42, 43).

Other studies have utilized fossil evidence calibrated to molecular evolution rates to estimate divergence of species. Using mitochondrial DNA, Redeye Bass were shown to be basal to most other species of *Micropterus* with an evolution in the late Miocene 8-10 million years ago (mya) (38). A later study included nuclear DNA and updated the fossil used to a more accurate Centrarchid ancestor, with similar results except that now Shoal Bass are basal to most other black basses. Also, the timing of the diversification within this genus was correlated to a period when there was a known global climate change (44). It is important to note that Alabama Bass were not included in either of those studies. In 2021, Alabama Bass were included in the analyses using nuclear DNA to examine phylogenetic relationships among the black basses. The results show discordance when compared to the mitochondrial results in the same groups, indicative of a history of introgression. These data show that Redeye Bass evolved around 3 mya, a couple of million years prior to Alabama Bass. Interestingly, Redeye Basses were monophyletic and distantly related to Alabama Bass. These evolution times were also consistent with sea level changes during the Pliocene and Pleistocene that led to geographic isolation and speciation (45). A recent study, using the most robust and powerful genomic methods to date, confirmed the late Pliocene/early Pleistocene as the era when most speciation occurred. All of the Redeye Bass were shown to have moderate to high genealogical divergence index (gdi) values supporting considerable genetic divergence within that group and strong evidence for validation of their species status (46).

3. Redeye Bass

3.1 Species and Distribution

First described in 1940 by Hubbs and Bailey, Redeye Bass are native to upland streams in the southeastern United States (6, 30). Aspects of Redeye Bass and their distinctions relative to Smallmouth Bass were published as early as 1877 (47), but the species had never been formally described (30). Hubbs and Bailey did note that Redeye Bass observed from the Black Warrior River system may be a distinct subspecies due to morphological differences not present in other Redeye Bass from the MRB (30). Despite their description over half a century ago, Redeye Bass have been one of the least studied groups within the black basses. In 2013, Redeye Bass were formally described as five different species based on genetic and morphological differences, each restricted to a unique river system (6). Redeye Bass are native to the Coosa, Cahaba, Black Warrior, and Tallapoosa River systems of the MRB, the Chattahoochee River system, and the Savannah and Altamaha River systems of the Atlantic Slope (6, 43). Populations of Redeye Bass in the Savannah and Altamaha systems are diagnostic by morphological and genetic traits (42, 43) and have been shown to merit species status (43, 46), but they are still awaiting formal description. In the MRB, the distribution of Redeye Bass overlaps with that of Alabama Bass, Redeye Bass are generally considered an obligate lotic fish, preferring the cool, rocky waters of upland streams above the Fall Line, the transition between the upland physiographic provinces to the Coastal Plain (6, 30, 48). Redeye Bass are fluvial specialists while Alabama Bass are a species generalist that can inhabit both lentic and lotic habitats (6, 30, 41, 49). Although Redeye Bass in the Savannah River system, commonly referred to as Bartram's Bass, do persist in reservoirs (50, 51). Each species maintains allopatry from one another due to larger mainstem habitats or by drainage divides (6). Given the limited native range of these species, they are vulnerable to extinction and recent surveys have already suggested a distributional decline in the Black Warrior River system (6, 9, 52).

Redeye Bass have been introduced outside of their native range across the United States to create a fishing opportunity and to control rough fish populations in small foothill streams that are too cold for other bass and too warm for trout (53). In 1954 they were collected from a tributary to the Conasauga River and transported to California where they were raised in a hatchery and introduced to the following streams in the early 1960s: Alder Creek, South Fork of the Stanislaus River, Sisquoc River, Santa Margarita River, and Dry Creek (54). Redeye Bass from the Coosa River system were also introduced at some point to the upper Verde River system in Arizona where they still persist today. Until recently, they were thought to be Smallmouth Bass, and have been managed as such for the last six decades (55). More locally, Redeye Bass have been introduced to Cumberland river systems in Tennessee where they still persist today (56).

3.2 Habitat and Biology

To better conserve and manage these recently described Redeye Bass, an understanding of environmental and habitat factors related to occurrence and abundance is needed. Redeye Bass prefer rocky substrates and vegetative cover (57) and have been detected more often in areas in with high rock scores relative to those with lower rock scores (58-60). The rock scores increase with size and complexity, and both the amount of rock and complexity of rock substrate was significantly and positively associated with increased Redeye Bass presence (58). These habitat types are common in the Piedmont physiographic province and likely contributes to the association of Redeye Bass occurrence and abundance in this region

(58, 61). Redeye Bass may utilize these areas for concealment to ambush prey and as a refuge from periods of increased water velocity. Additionally, complex rocky substrate could provide food resources such as higher concentrations of invertebrates (62). Other metrics such as stream size have been correlated with higher numbers of Redeye Bass (51), indicating their preference for smaller streams (61). Stream gradient has also been shown to be an important habitat criteria for Redeye Bass, which is another feature common to Piedmont streams (4, 58, 61), which may be related to their preference for cooler water temperatures and better dissolved oxygen levels, which also increase the abundance of prey (63).

Much of the basic biology and life history traits of this species are poorly understood due to a lack of studies investigating them. Redeye Bass are the slowest growing black bass and rarely reach sizes greater than 12 inches in total length and a pound in weight in streams (48, 64, 65), but they do grow faster in the reservoirs of the Upper Savannah River system (66). They tend to grow fastest in their first year (48, 65) then grow an inch per year (48). Other studies with substantial number of Redeye Bass collected report sizes ranges from 50 to 300 mm (N=407) (58) and 30 to 300 mm (N=530) (60), indicating the relatively common small size range of these fish across their native distribution. Notably, those size estimates may have been confounded by hybrids as they did not include genetic confirmation of pure fish. Relative to other black basses, their growth is slower, but their lifespan is average (48). There are no comprehensive studies dedicated to understanding the food habits of Redeye Bass, but a few studies have examined diet as part of a larger study. A study in the upper Coosa River system of Tennessee found that the most common diet items found in the stomachs of Redeye Bass were adult aquatic and terrestrial insects, although this study was conducted during the summer months when these food items would have been abundantly available (48). A more recent study on the Tallapoosa River found that crayfish were the dominant prey item by weight in the winter, while insects and insect larvae predominated in the spring and fall. Summer months consisted of primarily insects and crayfish with minimum observations of piscivory (67).

Despite the inclusion of “red eye” into the common name, eye pigmentation is not a reliable diagnostic characteristic for Redeye Bass as the color can change within seconds due to stress and temperature. All Redeye Bass do have a silver to blue crescent located on the posterior half of the upper eye that is diagnostic (6). Additionally, the presence of white margins on the upper and lower lateral margins of the caudal fin are diagnostic morphological features of the Redeye Bass species group (6). Both of these diagnostic features can be confounded by hybridization with sympatric Alabama Bass, with intermediate features of both parental form conflated with intraspecific variation (6). All Redeye Bass have a tooth patch, although it is considerably smaller or absent in the Redeye Bass from the Black Warrior (6). Each species of Redeye Bass is further distinguished by variations in lateral body pigmentation patterns and fin coloration were observed changing rapidly due to stress and water temperature (6, 43).

There is a paucity of available literature concerning the spawning habits and requirements of Redeye Bass. One study showed that they spawn in late May and June, with observations of shallow depressions in coarse cobblestone in the head of pools (48). May and June have been linked to higher counts of Redeye Bass and the presence of more vibrant colors assumed to be associated with spawning (58, 68). It is believed that some migration occurs during spawning season as Redeye Bass have been observed moving downstream when temperatures begin to cool in the fall, then back into the tributary stream in early spring (48). The early spring migrations were composed of primarily adult fish and involved navigating over a six-foot vertical rock dam (48). Results from tagged fish in the Tallapoosa River system confirmed long migrations (20 km) of Redeye Bass in the spring, assuming an association with spawning (68). A later study observed Redeye Bass nests in slower water near the bank at variable depths with nests utilizing a variety of substrates. The more important microhabitat features were shown to be slower water velocities downstream from a major flow and distance from the bank or refuge habitat rather than substrate or depth. There also appeared to be some site fidelity in nest locations from year to year (69).

3.3 Ecological Value

Redeye Bass are ecologically valuable as a top predator in their small upland streams (14). They also serve as general and specific fish hosts to numerous mussel species, another extremely biodiverse and imperiled taxa in the MRB (70). Gravid female mussels have adapted their mantle flaps and other structures to mimic food items for fish such as a small fish or large invertebrate. When the bass attempts to eat the "lure", they become infected with glochidia, or the larval stage of mussels (71). The larvae eventually metamorphose into juveniles and drop off the fish host starting their benthic lifestyle (72). Redeye Bass serve as fish hosts for the Finelined Pocketbook (*Lampsilis atilis*), Plain Pocketbook (*Lampsilis cardium*), Orangenacre Mucket (*Lampsilis perovalis*), Alabama Rainbow (*Villosa nebulosa*), and the Southern Rainbow (*Villosa vibex*) mussels (70-72).

3.4 Economic Value and Sportfish Management

The Redeye Bass was described by H.S. Swingle as “highly regarded by local fishermen because of their gameness” (30). Redeye Bass live in waters typical of another sportfish, the Brook Trout (*Salvelinus fontinalis*), that are characterized by waterfalls, bedrock boulders, and clear water. These similarities with Brook Trout in size and habitat preferences have resulted in the Redeye Bass being referred to as the “brook trout of the warm-water game fish” (48). The Redeye Bass boasts colors not found in other any other bass (6), but it’s small size and limited distribution have limited its popularity among anglers. Recently, the pursuit of Redeye Bass has gained interest among fly anglers and has developed a loyal following of anglers interested in the conservation of these unique sportfish (73). In essence, this species is being utilized as a driver of watershed-level conservation by developing an appreciation of its intrinsic value within its native habitat and ecosystem. The fish are particularly wary in small streams offering a challenge to even the most experienced anglers. When hooked, the fish are “scrappy, colorful, and palatable” (48). Due to their slow growth and vulnerability to angling (65), a stream could easily be impacted by too much fishing pressure which merit a possible evaluation of the current daily bag limits for black bass in all states harboring native populations of Redeye Bass.

4. Hybridization

4.1 Susceptibility of Black Basses

Hybridization is more commonly observed in fishes than any other vertebrate group (74, 75) and is considered to be one mechanism vertebrates use for diversification (76). Hybridization is defined as the reproduction between members of genetically distinct populations (77) and is generally referenced at the species level (78). A reliance on external fertilization, weak reproductive barriers, unequal abundance of parental species, and decreasing habitat complexity render them susceptible to hybridization events even among divergent taxa (74, 79, 80). The black basses are no exception to this as hybrids among every taxa have been observed (81), many of which persist to contribute to future generations suggesting heterospecific crosses in fish results in reduced developmental incompatibilities than other vertebrates of similar genetic divergence (79). While natural hybridization can be an important avenue of gene flow between populations to adapt to changing conditions by providing novel genotypes and epistatic interactions (82), it is thought to be rare among the black basses (38, 42). Species of bass have traits that range from color and courtship behavior that work in concert to limit hybridization (83). However, due to the popularity of black bass among anglers there has been widespread translocations to create and enhance fishing opportunity (16), which has resulted in human-mediated hybridization (50, 56, 84-86). Hybridization can result in hybrid offspring that are less fit than parental forms and selected against, but if hybrid offspring survive and are able to contribute alleles to future generations, it is termed introgressive hybridization (87). Introgressive hybridization between native and introduced species can lead to the creation of hybrid swarms, bimodal hybrid zones, and complete replacement of native species (51, 56, 85, 86, 88, 89). Hybridization among the black basses threaten to homogenize the existing biodiversity in this ecologically and economically important genus, much of which is still being discovered and described (25, 46).

One reason that *Micropterus* spp. hybridize so readily is due to their relatively low divergence, allowing for hybrid viability that is not commonly observed in other animal species (44). Knowledge that reproductive isolating mechanisms are incomplete allowing for fertile hybrids necessitates preservation of black bass gene pools through conservation and management. It is thought that black bass lack distinct sex chromosomes and the predominance of males means that hybrid males would have to backcross with pure females for introgression to occur (44, 90). This has been observed in at least one hybridizing population where female Redeye Bass mated with male Alabama Bass, indicative of unidirectional hybridization resulting in widespread introgression (42). This is especially important in rare and vulnerable species such as the Redeye Bass as introgressive hybridization could result in extinction.

4.2 Genetic Tools and Management of Hybridization

It is impossible to discuss hybridization among the black basses without mentioning the methods by which hybridization has been examined. Historically, hybridization was identified by meristic and morphological characteristics until the late 1960s when the advent of genetic markers allowed for increased accuracy in the detection of the extent and direction of hybridization (91, 92). This development overcame the complications of identifying hybrids past the F1 generation, ontogenetic differences in morphology and color, and the difficulty in assessing the extent of hybridization (77, 79). Allozymes allowed for individual-level assessment of most species using protein coding genes as biochemical markers (74, 79, 93), but were limited due to their limited abundance making a determination of the extent of hybridization difficult (56). Allozymes solved some of these issues and predominated in usage through the 1980s when mitochondrial DNA was added as a complimentary investigative tool (56, 94, 95), providing a new perspective on the genetic structuring present in natural and hybridizing populations due to its maternal inheritance and lack of recombination (96). The late 1990s and early 2000s ushered in a new era of nuclear DNA techniques based on widely distributed repetitive elements, known as microsatellites, that began to rise in popularity (79) over allozymes due to their increased variability and ability to assess a large number of polymorphic loci in almost any species (96). Microsatellites are repetitive elements that continue used widely in species classification and identification of hybrids (85, 97) that offered. Today, single nucleotide polymorphisms (SNPs) dominate the literature relating to hybridization and tools to detect hybridization (98-100), due to their cost-effectiveness and abundant distribution in the genome (101). These loci are located in both coding and non-coding regions of the genome and allows for more power to investigate genetic and population metrics such as gene flow and heterozygosity levels (96). The benefits of combining mitochondrial DNA analyses and morphometrics with nuclear techniques are recommended as a wholistic approach to answering complicated questions (25, 43). As these technologies become more accessible for fisheries scientists, genetic methods continue to rise in appreciation among fishery science, especially as it relates to conservation, management, aquaculture, and evolutionary biology (79).

From a management perspective, some state agencies are conducting reservoir and stream-based surveys of purity and hybridization among black basses, while others focus only on reservoirs, and yet still some have no baseline genetic surveys. The ability of genetic surveys to inform management decisions ranges from locating pure populations to establish conservation plans (Thongda et al. 2019) and highlighting populations that are experiencing bottlenecks or low levels of heterozygosity (Taylor et al. 2019; Taylor et al. 2018). One of the most studied black bass species is the Largemouth Bass. Despite evidence supporting that there are two separate species of Largemouth Bass (Bolnick and Near 2005; Kassler et al. 2002; Near et al. 2003), there is no unified acceptance of this across all agencies and the American Fisheries Society. Many state agencies focus their sampling regimes on population characteristics of Largemouth Bass in reservoirs to monitor the efficacy of stock enhancement from the introgression of Florida Bass alleles into Largemouth Bass populations. Unfortunately, many management agencies have little concern for the dangers of introgression with non-natives and continue to stock Florida Bass into native Largemouth Bass waters to produce trophy fisheries with little regard of the science that exercises caution in these situations (Kassler et al. 2002) to maintain native biodiversity. Conversely, the Florida Fish & Wildlife Conservation Commission (FWC) prohibits the stocking of Largemouth Bass into their waters to protect native Florida Bass populations in their Black Bass Management Plan, the most comprehensive of its kind in the country. Although the Largemouth Bass reigns supreme to sports fishers and therefore to fishery managers, increasing attention is being placed on the diversity of black basses. Owing to their limited distribution, very little known about many of the endemic black basses. Texas has completed a range wide survey for their state freshwater fish, the endemic Guadalupe Bass, after almost losing them forever to hybridization with introduced Smallmouth Bass (Bean et al. 2013). The stream dwelling black basses, such as Guadalupe Bass, have the potential to be economically valuable to states. Texas Parks and Wildlife showed that over $71 million and over 700 jobs were created due to river and stream angling in Texas, with almost 50% of those anglers pursuing the Guadalupe Bass (Thomas et al. 2015). Research is ongoing to better characterize Redeye Bass populations in the Mobile Basin due to hybridization with native congener Alabama Bass, which has resulted in lack of scientific consensus regarding the species status of that group. Other populations of yet to be described Redeye Bass are species of special concern due to introgressive hybridization with introduced Alabama Bass (Baker et al. 2013; Freeman et al. 2015). Shoal Bass are still being studied in Georgia to better characterize genetic structure, hybridization threats, and habitat loss (Taylor et al. 2018), but the species has been functionally extirpated from Alabama streams. Little is still known about the Suwannee Bass genetics, or the yet to be described Choctaw Bass (Tringali et al. 2015).

4.3 Hybridization Among the Black Basses

The literature is full of examples of hybridization among the black basses, all of which involve human-mediated hybridization due to translocations (24, 51, 56, 85, 88, 89). Many instances of hybridization remain undetected due to lack of genetic surveys by state fish and game agencies despite the high morphological similarities known to exist in introgressed populations (56, 81, 102).

Genetic studies investigating hybridization in Texas among Guadalupe Bass and other introduced black basses were some of the first to document the interspecific hybridization occurring between formerly allopatric species (86, 103). Guadalupe Bass are endemic to central Texas and hybridization has reduced them to only a few genetically pure populations (86). Introgressive hybridization was also documented between Smallmouth Bass and Spotted Bass in the Missouri River System (104). Redeye Bass were introduced to over a dozen streams in the upper Cumberland region of Tennessee, within the native Smallmouth Bass range, during the mid 1950s to enhance fishing opportunities in headwater streams. A few decades later, these introductions resulted in 66% of samples collected being composed of Redeye Bass X Smallmouth Bass hybrids (56), illustrating the speed at which hybridization can affect populations.

Although many species of bass have been translocated to enhance sportfishing opportunities, the spread of Alabama Bass by anglers and its deleterious impacts to native biodiversity have been paramount in the last few decades. Alabama Bass were introduced into the Chattahoochee

River system in the 1980s which have subsequently hybridized with native Shoal Bass and Chattahoochee Bass in addition to hybridization with other introduced congeners (88, 105). Lake Chatuge in Georgia was historically a phenomenal Smallmouth Bass fishery until the introduction of Alabama Bass transformed it into an Alabama Bass fishery through genetic swamping in just over a decade (24). North Carolina has also experienced a change in native species abundance due to the introduction of Alabama Bass. Lake Norman was once a popular fishery for Largemouth Bass until the introduction of Alabama Bass flipped the composition of the black bass fishery into an Alabama Bass fishery (89, 102). More recent genetic surveys have revealed extensive immigration of Alabama Bass into river systems of North Carolina and Virginia, extending their invasion northward (Personal Communication, Scott Loftis, NCWRA; John Odenkirk, VADGIF). The lack of fish and game agencies performing routine genetic surveys limit the accuracy of the true extent of Alabama Bass introductions and subsequent hybridization consequences. Although the negative impacts due to Alabama Bass introductions continue to be well documented and researched.

4.4 Hybridization Between Alabama Bass and Redeye Bass

Hybridization is well documented between Alabama Bass and Redeye Bass in systems where one species is introduced (50, 106), although much work is still needed to elucidate why these two species hybridize so readily. Anglers illegally introduced non-native Alabama Bass in the mid-1980s to the Keowee Reservoir of the Savannah River system, which contains the endemic Bartram's Bass (*Micropterus sp. cf. cataractae*) (Barwick et al. 2006; Freeman et al. 2015). A decade later, Alabama Bass became the most frequently caught sportfish on Keowee Reservoir. This four-fold increase in Alabama Bass catches was correlated with an 83% decline in catch rate of Bartram's Bass, suggesting the two might be hybridizing. Nuclear and mitochondrial DNA analyses confirmed that not only is hybridization occurring between Bartram's Bass and Alabama Bass in Keowee Reservoir, but it appears to be occurring unilaterally between Alabama Bass males and Bartram's Bass females (Barwick et al. 2006; Oswald 2007). By 2007, there were no pure Bartram's Bass found in Lake Keowee. In addition, sampling three other major reservoirs for the Upper Savannah River system showed that Alabama Bass are present in Lake Jocassee, Lake Hartwell, and Lake Russell. Furthermore, Bartram's Bass X Alabama Bass hybrids were found in all three of the systems described above (Oswald 2007; Bangs et al. 2017). Given that only two of these systems are known to have introductions of Alabama Bass, this highlights the dangers of translocating fish and their ability to quickly spread into other systems. This is especially concerning given that the Upper Savannah River system constitutes a major portion of the native range of the geographically restricted, and currently undescribed, Bartram's Bass. The tributary populations of Bartram's Bass in the Upper Savannah River system, which are presently some of the only pure populations remaining (Leitner et al. 2015), are being prioritized for conservation.

To further complicate matters on the Savannah River, in 2007 non-native Smallmouth Bass (*Micropterus dolomieu*) were illegally introduced to the lower Savannah River in the shoals area near Augusta, GA. A sampling of the fish in the river around this time showed no non-native alleles present. After sampling fish a few years later, 30% of fish sampled were hybrids between native Bartram's Bass and non-native Smallmouth Bass. There has not been a sampling conducted since then, but there are plenty of examples of non-native congeners overwhelming native populations, so the outlook does not look promising (Birdsong et al. 2010).

Hybrids between sympatric populations of Redeye Bass and Alabama Bass have been observed in the MRB (6, 30, 41). In 1940, Hubbs and Bailey proposed that hybridization has played an important role in the evolutionary history of both Redeye Bass and Alabama Bass and that Alabama Bass might have recently immigrated into the range of Redeye bass (30). Previous studies have shown that Alabama Bass and Redeye Bass are sister taxa (15, 43, 46), owing their relationship to historical hybridization between the two overlapping species (15, 46). More recent studies show that Redeye Bass evolved prior to Alabama Bass around three million years ago and are distantly related to Alabama Bass (45), with the discordance between nuclear and mitochondrial phylogenies indicative of a high degree of introgression in the evolution of these species (107). Although there has been some work examining phylogenetic relationships, much work is still needed to document and investigate hybridization between these two species in their native MRB.

4.5 *Anthropogenic Hybridization*

North American landscapes have been widely altered since the arrival of the first Europeans. The prevalence of agricultural land use is the primary land use in many watersheds across the southeastern US which also contains the heart of black bass diversity (25). Impacts on watersheds from agricultural land-use practices range from excess sedimentation and nutrient enrichment to the alteration of riparian areas, all of which can work independently or in concert to reduce the habitat quality of adjacent waterways (108). The reduction and alteration of habitat required by certain species can have the same confounding effects on sympatric populations as the secondary contact of formerly allopatric species (81). Little is known about the exact relationships between anthropogenic disturbance and its effect on Redeye Bass, although many studies show a negative trend between habitat disturbance and presence of Redeye Bass. Shoal Creek in the Coosa River system showed a decrease in Redeye Bass due to habitat alteration by an impoundment creating permanent lentic habitat in a 5.5 km section (65). Streams impacted by anthropogenic disturbances have been shown to shift fish assemblages to more tolerant and generalist fishes while streams surrounded by heavily forested land cover has a more diverse fish assemblage composed of more specialists (109). The removal of habitat-specific niches can enhance the survival of hybrids which can exacerbate the effects of hybridization (75, 110). Relative abundance of hybrids has been positively correlated with anthropogenic habitat disturbances such as increased agricultural land use (110, 111) while occurrence of Redeye Bass have been associated with more forested and high elevation watersheds in Piedmont streams with their occurrence affected by levels of development (69) and more readily detected in areas with lower levels of disturbance at both the local and watershed scale (112) indicating sensitivity to habitat changes.

Hydroelectric dams and mill dams were constructed throughout many waterways in the Piedmont and Ridge and Valley ecoregions due to the potential energy provided by their high gradient water systems (113, 114). The construction of the dams restricted and fragmented fluvial species and destroyed lotic habitat allowing for generalist black bass species to proliferate through creation of lentic habitat and non-native introductions (16). Surveys have been recommended in the Black Warrior River system due to a distributional decline of Redeye Bass (6), and a later survey confirmed the decline of Redeye Bass due to the replacement by Alabama Bass as a result of habitat alteration from the construction of Lewis Smith Reservoir (52). Dam construction has altered lotic habitats preferred by Redeye Bass and reduced connectivity between populations that may have deleterious effects on genetic diversity (105). A more recent study in the Black Warrior River system showed that Redeye Bass were negatively associated with higher levels of disturbance, and almost completely absent from areas central to the Warrior Coal Field (60), which has been heavily mined since the 1800s (115). The highest numbers of Redeye Bass from the Black Warrior system were detected in the heavily forested Bankhead National Forest (60), suggesting the increased likelihood of occurrence of Redeye Bass in areas with low levels of disturbance indicates that habitat disturbance negatively affects Redeye Bass. This information on habitat preferences and factors that negatively affect Redeye Bass populations should be incorporated into the management and conservation plans of these important species.

Anthropogenic activities such as agriculture, pollution, urbanization, altered temperature regimes, and mining on a landscape scale can promote hybridization by affecting the ecological integrity of a stream and reducing stream heterogeneity (108), weakening reproductive barriers (108, 111), and through increased pollution (116, 117). Human-mediated disturbances such as translocations and habitat fragmentation can exacerbate and compound these factors and facilitate hybridization between native species (118, 119). This human-mediated hybridization can have deleterious effects on local biodiversity by causing erosion of native genomes, outbreeding depression, genetic swamping, and extinction (51, 79) and is considered to be one of the primary threats to freshwater ecosystems (120).

Agricultural land use can elevate turbid conditions in lotic systems that promote interspecific interactions. In addition to increased sedimentation, nutrient pollution from agricultural practices have resulted in numerous major fish kills in adjacent waterways (121, 122). The presence of hybrids between naturally sympatric Red Shiners (*Cyprinella lutrensis*) and Blacktail Shiners (*C. venusta*) has been linked to turbid conditions resulting from excessive sedimentation in the

Guadalupe and San Marcos rivers of Texas (123), with abatement of hybridization in these systems as water clarity improved (124). Increased turbidity can affect prezygotic isolating mechanisms by eliminating visual recognition and assortative mating cues (90, 125, 126). The scarlet-colored operculum fringe of Redear Sunfish (*Lepomis microlophus*) has been shown to play a role in species recognition and preventing hybridization with Bluegill (*L. microchirus*). Once removed, by physical removal and obscurement due to turbid conditions, the two species readily hybridized (90). These experiments illustrate the importance of color and visual recognition as a reproductive isolating mechanism in the sunfishes and indicates a potential role for how habitat alteration and other anthropogenic factors could relax sexual selection between genetically distinct species of *Micropterus* spp.

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Chapter II Genetic evidence of introduced Coosa Bass (*Micropterus coosae*) and Alabama Bass (*M. henshalli*) and hybridization with native Micropterus spp. in Town Creek, Alabama, USA

Abstract

The black basses (*Micropterus* spp.) are some of the most important game fishes in the United States. Translocation by both anglers and state agencies for increased angling opportunity has led to widespread hybridization between native and non-native species. The use of species-diagnostic single-nucleotide polymorphism (SNP) markers for the black basses have allowed for rapid assessment of species identification and hybridization in rivers and streams. Here, we report genetic evidence for the presence of Coosa Bass (*Micropterus coosae*) and Alabama Bass (*Micropterus henshalli*) outside of their native range and their hybridization with native species of black bass in Town Creek, a tributary of the Tennessee River. To determine which species of bass were present in the creek, four sites were sampled using both angling and backpack electrofishing equipment. DNA from fin clips was analyzed using a diagnostic SNP panel. Genetic results confirmed that Coosa Bass and Alabama Bass were present as non-native congeners in the creek. Interestingly, no pure individual Smallmouth Bass (*M. dolomieu*) or Spotted Bass (*M. punctulatus*) were found, although both species are native to the stream. Phenotypic identification followed by genotypic classification revealed that 62% of the fish collected for this study were mis-identified in the field, with 100% of those being hybrids, indicating the importance of genetic assessment in conjunction with classic morphometrics for management of black basses. Further studies are needed to determine if seasonality plays a role

in species presence and abundance and whether population profiles identified here are observed more broadly within the Tennessee River system.

1. Introduction

The black basses (*Micropterus* spp.) are one of the most popular freshwater game fish in the United States (1) and are an incredibly diverse taxa (2). The desire for enhanced angling opportunity has led to translocation of some *Micropterus* spp. that were previously geographically isolated. These widespread movements have resulted in inter-specific hybridization accelerated by habitat alterations and weak prezygotic isolating mechanisms (3-7). The hybridization effects are often detrimental to native species through introgression of non-native alleles leading to, in some cases, rapid loss of native biodiversity (5, 7, 8). Hybrid black basses have demonstrated, in some cases, a reduced fitness resulting in poor adaptation to their environment (9, 10), but in others, a hybrid vigor that facilitates rapid invasion (11-13).

The Mobile River Basin (MRB) is home to the highest number of endemic black bass species of any basin in the world, including Largemouth Bass (*M. salmoides*), Alabama Bass (*M. henshalli*), and four described species of Redeye Bass: Coosa Bass (*M. coosae*), Cahaba Bass (*M. cahabae*), Tallapoosa Bass (*M. tallapoosae*), and Warrior Bass (*M. warriorensis*) (14, 15). Unfortunately, these species have been commonly translocated to surrounding basins outside their native ranges. However, our knowledge of these introductions has often been limited to surveys focused on larger rivers and reservoirs. In smaller streams, introductions and resulting hybridization may go overlooked due to infrequent sampling and difficulty of access. In such systems, angler accounts and accompanying photographs are often valuable indicators of the presence of non-native species. In this vein, angler reports and video evidence indicated that two species native to the MRB, Coosa Bass and Alabama Bass, were likely present in Town Creek, a tributary to the Tennessee River in Marshall and DeKalb counties in Alabama.

Alabama Bass have been introduced to many waterways in the southeastern US resulting in numerous negative interactions with native black basses. For example, Lake Chatuge and Lake Blue Ridge in Georgia have lost the vast majority of their native Smallmouth Bass populations due to replacement by hybridization and introgression with introduced Alabama Bass (5). Similarly, the Keowee reservoir of the upper Savannah River was dominated by Alabama Bass less than a decade after their initial introduction. The concomitant decline of native Bartram's Bass (*M*. sp. cf. *cataractae*) was shown to be the result of hybridization and introgression rather than competition for resources (7, 8). The Chattahoochee River has also experienced illegal introductions of Alabama Bass that have threatened endemic populations of Shoal Bass (*M. cataractae*) (7).

Coosa Bass have also been stocked into areas outside of their native range to establish a fishery in streams that are too cold for other black bass, but too warm for trout (16). Coosa Bass were stocked into several inland and coastal streams in California to create a fishing opportunity (17) where they have had deleterious effects on the native fauna in those streams (18). The introduction of Coosa Bass into the Upper Cumberland Region of East Tennessee is documented to have occurred in the mid-1950s in 16 streams among the Tennessee and Cumberland River systems. Subsequent allozyme analysis revealed widespread hybridization occurring between the native Smallmouth Bass (*M. dolomieu*) and introduced Coosa Bass. The effects of hybridization extended beyond those streams initially stocked, indicating the potential for migration and/or additional, undocumented introductions (4).

Given the potential for adverse effects of black bass introductions outside their native ranges, regular sampling and surveillance of smaller streams are needed to detect and potentially identify areas of conservation concern. Based on angler reports, we hypothesized that Coosa Bass and Alabama Bass were present in Town Creek, Alabama and that hybridization is occurring, but undetected based on traditional morphological assessments. Our study, therefore, utilized recently developed single-nucleotide polymorphism (SNP) panels for black basses (19) to evaluate angled and electrofished samples from Town Creek, characterizing the genetic composition of the black bass community in this system. We also compared the concordance of phentoypic assessment when compared to genotype results in our samples involving potential cryptic hybrids. Our results demonstrate a conservation need to better document and understand genetic characteristics in black bass communities.

2. Methods and Materials

2.1 Sample Collection

Sample sites were determined by available public access points along Town Creek. The site furthest downstream (Site 1) was near the confluence with Lake Guntersville at the Town Creek Fishing Center. A 4.6 m aluminum boat outfitted with a Smith-Root 5.0 GPP electrofishing system traveled upstream on Town Creek to the first set of impassible rocks (latitude 34.429941, longitude -86.124236) and used 4-6 amps at 120 pulses per second of direct current (DC) to sample all available habitat in the immediate area (July 2019). The next site was 8.5 km (river kilometers) upstream at High Falls Park (latitude 34.399930, longitude -86. 066689) and due to water depth, only angling was used to sample this site immediately below the falls (July 2019). The final two sites were 6 km upstream at the Highway 227 bridge crossing (latitude 34.3900589, longitude -86.019641) and another 17.5 km upstream at the County Road 50 bridge crossing (latitude 34.427362, longitude -85.876316). Both of these sites were sampled immediately upstream and downstream of bridges using angling and two Smith-Root LR 24 electrofishing backpacks (350-400 V, 750 seconds, 60 pulses per second) (July/August 2019). Directed sampling was used to target all available habitat types (pools, runs, and riffles) in an attempt to reach a target number of samples from each site. As such, there was no standardization by time, or length of stream.

All black bass individuals were netted, measured to the nearest total length (mm), weighed (g), and phenotypically identified to species and recorded as Alabama Bass (ALB), Largemouth Bass (LMB), Coosa Bass (CSB), Smallmouth Bass (SMB), Spotted Bass (SPB), or hybrid (Table S1), photographed for phenotypic record, and fin clipped for genetic analysis. Fin clips were placed into 95% ethanol (EtOH) for storage and transport.

2.2 SNP Genotyping

Genomic DNA was extracted using a simple sodium hydroxide (NaOH) and hydrochloric acid (HCl) tissue digestion (20). Genomic DNA was quantified using the Nanodrop 2000c spectrophotometer (ThermoFisher, Waltham, MA, USA). Samples were genotyped using a 64-

SNP panel designed previously from genotype-by-sequencing (GBS) data for classification across six to nine *Micropterus* species (19). Amplified and mass-specific extension reactions (iPLEX™ Gold Assay) were performed using 2 µL of input DNA at concentrations ranging from 34-351 ng/µL (21). Genotypes were generated using the MassARRAY (Ageno Bioscience Inc., San Diego, CA) system according to manufacturer's protocols. SNP genotyping calls were generated automatically using the MassARRAY Typer 4.0 Analysis software. All samples were processed with a positive control of Smallmouth Bass DNA with known genotype and a total of 5 individuals were run on multiple plates with 99.6% of genotypes matching among replicates (data not shown).

2.3 Data Analysis

Genotype data were analyzed to identify species of the *Micropterus* samples from the Tennessee River system in Alabama using a Bayesian clustering algorithm-based program, STRUCTURE version 2.3.4 (22). The STRUCTURE runs were performed assuming six populations (K=6), as six of the more common reference *Micropterus* species' genotypes were included in each analysis (Alabama Bass (*n*=70), Largemouth Bass (*n*=70), Coosa Bass (*n*=60), Shoal Bass (*n*=70), Smallmouth Bass (*n*=70), and Spotted Bass (*n*=70)) for resolving taxonomic and hybridization status of each individual. Thongda et al. 2019 optimized the 64-plex black bass panel for use with these 6 species; note, the utilized SNP marker panel does have limitations in differentiating within the Mobile Basin Redeye Bass group. While Coosa Bass was used as a reference species for this study, it is possible it could be another member of the Redeye Bass group given the continuing scrutiny and study of this group. Shoal Bass were chosen as a reference species over other black basses, such as Suwannee Bass (*M. notius*) and Choctaw Bass

(*M. haiaka*), based on their geographical proximity to the sample sites. STRUCTURE analyses were performed using the admixture model (K=6) in conjunction with the USEPOPINFO and POPFLAG models with a migration prior set to 0.01 with correlated allele frequencies and a burn-in of 20,000 iterations followed by 200,000 repetitions of Markov chain Monte Carlo (MCMC) simulation (23). STRUCTURE runs were performed in triplicate using the R package *strataG* (24) with the Q-values averaged across replicates. STRUCTURE results with individual genomic proportion values (Q-values) of ≥0.95 were assigned to a single species ("pure"). For hybrid individuals, a Q-value threshold of ≥0.05 was required to be considered a contributing species proportion.

3. Results

3.1 Sample Collection

In Town Creek, we collected 83 black bass specimens (mean TL = 162 mm; SD = 81 mm) from four sampling sites (Table 1). Phenotypic identification at Site 1 produced 8 Largemouth Bass, 5 Spotted Bass, and 1 hybrid; 4 Coosa Bass, 2 Smallmouth Bass, 1 Largemouth Bass, and 4 hybrids at Site 2; 13 Coosa Bass, 6 Largemouth Bass, 2 Smallmouth Bass, 7 hybrids, and 1 specimen that was not identified in the field at Site 3; and 22 Coosa Bass, 6 Largemouth Bass, and 1 hybrid at Site 4 (Table S1).

Table 1 Locations of sample sites where specimens were collected for analysis of purity and hybridization from Town Creek, Alabama including site number, location description, GPS coordinates of sample site, mean total length (TL), standard deviation (SD), and number (N) of black bass collected from each site.

Site	Location	Latitude	Longitude	Mean TL (mm)	SD (mm)	N
1	Town Creek Fishing Center	34.4294842	-86.124359	210	99	14
2	High Falls Park	34.4002562	-86.068388	167	38	11
3	Highway 227 Bridge	34.3900589	-86.019641	128	46	29
4	County Road 50 Bridge	34.427362	-85.876316	133	75	29

3.2 SNP Population Genetics Analysis

Formal assignment of genomic proportions at K=6 of the 83 individual black bass samples from Town Creek in Alabama revealed substantial hybridization among five species belonging to the genus *Micropterus* at all four sites (Figure 1).

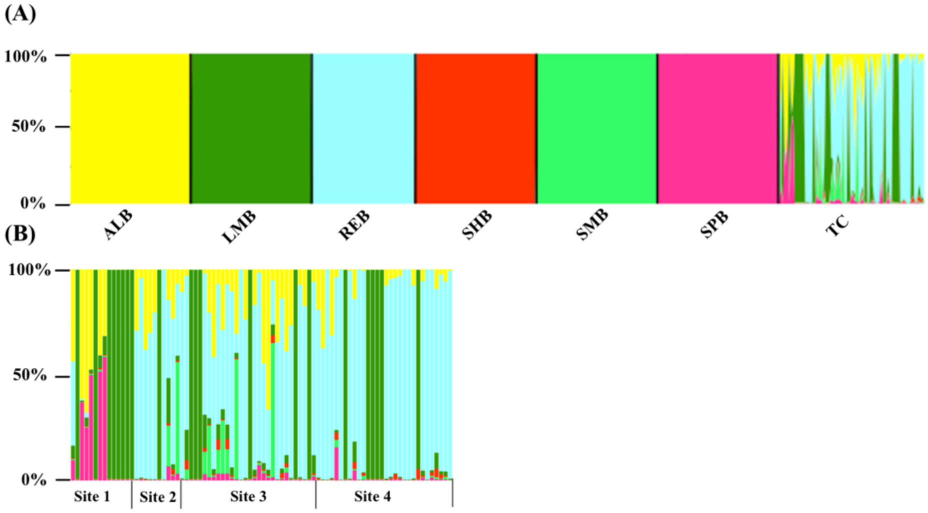

Fig. 1 a) Taxonomic assignments of individuals collected from Town Creek (TC) in Alabama and genotyped with 64 SNP markers diagnostic for six *Micropterus* spp. Assignments were made using membership coefficients for six (K=6) reference populations (ALB - Alabama Bass (*n*=70); LMB – Largemouth Bass (*n*=70); CSB – Coosa Bass (*n*=60); SHB – Shoal Bass (*n*=70); SMB – Smallmouth Bass (*n*=70); and SPB – Spotted Bass (*n*=70)). Distinct clusters are illustrated by different colors, with each individual's proportional assignment to those clusters represented by vertical bars. **b**) Enhanced image of TC individuals from a).

Genotype analysis revealed further differences in species composition between sites. Based on our sampling, pure Largemouth Bass constituted the majority of fish sampled at site 1 with the remaining percentage being composed of hybrid individuals. Moving upstream, sites 2 and 3 were comprised mostly of hybrids. Site 4 was comprised primarily of pure Coosa Bass and pure Largemouth Bass. The remaining individuals at Site 4 were classified as hybrids, with the most common being Alabama Bass X Coosa Bass (Table 2).

Table 2. Summary of taxonomic classification of black bass specimens collected from Town Creek, Alabama at each site, listed in order from downstream to upstream, based on genotyping with 64 SNPs diagnostic for six *Micropterus* spp. Classifications included are (ALB - Alabama Bass; LMB – Largemouth Bass; CSB – Coosa Bass; SMB – Smallmouth Bass; and SPB – Spotted Bass) with hybrids between any two or more species signified by a "X."

Species	Site 1	Site 2	Site 3	Site 4
ALB	0	0	0	0
CSB	0	2	1	14
LMB	8	1	6	6
SMB	0	0	0	0
SPB	0	0	0	0
ALB X CSB	0	6	13	6
CSB X LMB	0	0	1	0
ALB X SPB	3	0	0	0
CSB X SPB	0	0	1	1
ALB X CSB X LMB	0	0	1	2
CSB X LMB X SPB	0	0	0	0
ALB X LMB X SPB	2	0	0	0
ALB X CSB X SMB	0	1	3	0
CSB X LMB X SMB	0	0	1	0
ALB X CSB X LMB X SMB	0	0	2	0
ALB X CSB X LMB X SPB	1	0	0	0
ALB X CSB X LMB X SMB X SPB	0	1	0	0
N	**14**	**11**	**29**	**29**

Across all four sites, phenotypic identifications were often unreliable in light of subsequent genetic results. Overall, 62% of the fish collected for this study were mis-identified based on phenotypic characteristics. Phenotypic identification was only 64% accurate at Site 1, with Largemouth Bass being the majority correctly identified. Hybrid individuals had a higher degree of disparity between phenotype and genotype identification. Site 2 yielded a similar accuracy of phenotypic identification (64%) with genotypic hybrids being incorrectly recorded as pure species. Genetic assignment of individuals from Site 3 revealed a much lower accuracy for field identification (50%). Most misidentified individuals were again found to be hybrids of multiple combinations. Site 4, with the highest number of pure, unhybridized individuals, also had the highest percentage of correct field identification (72%) (Table S2).

The overall hybridization rate was 54% among the individuals collected from four different sites along Town Creek. The most frequently observed hybrid across study sites was between Coosa Bass and Alabama Bass (*n*=25), indicating the presence of two non-native congeners. Some individuals had predicted ancestry from four different species, suggesting a complex history of hybridization (Table 2). The only pure fish (Q-value ≥0.95) captured throughout the study were Coosa Bass and Largemouth Bass, with only the latter being native to this system. In all, 17 pure Coosa Bass were captured, with the majority coming from the uppermost collection site (Site 4), along with 21 pure Largemouth Bass (Table S1). Interestingly, in spite of a record of stocking (Table S3), no pure Smallmouth Bass were collected (Table 2). Furthermore, Smallmouth Bass genetic contributions were only detected in low amounts at Sites 2 and 3, and absent from the lower most and upper most sites. Overall, we observed Largemouth Bass in high abundance at the lowermost site (57%), but ranging from 9%-

20% of fish sampled at the three upper sites. Conversely, Coosa Bass (and their hybrids) were minimally present at the lower most site, but are the dominant group present at the upper three sites. Spotted Bass contributions were most abundant at the lower most site and equally present in small amounts at the other three sites. Alabama Bass alleles were present in equal amounts at the three lower sites while decreasing at the uppermost site (Figure 2).

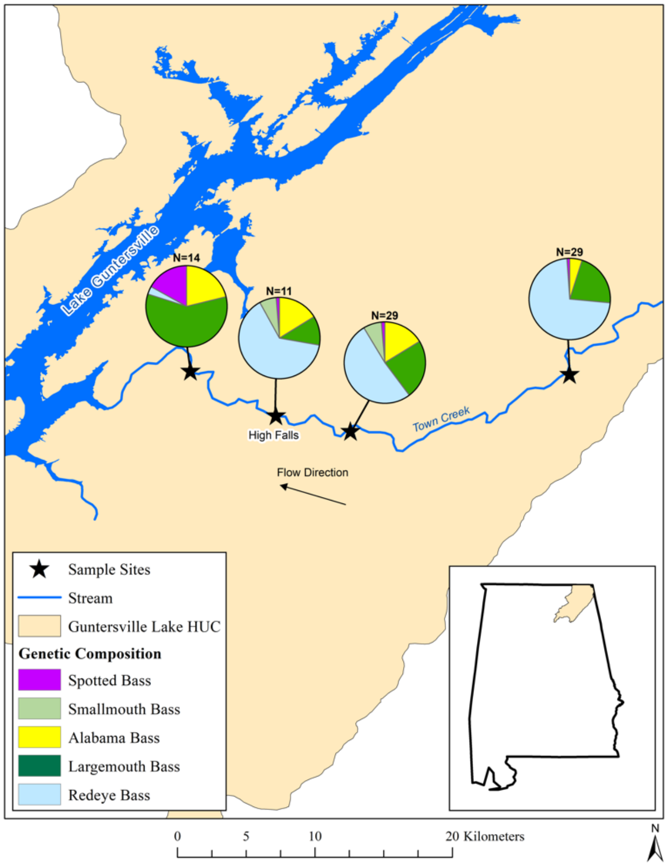

Fig. 2 Collection sites for black bass specimens from Town Creek genotyped using 64 SNP loci that are diagnostic for six *Micropterus* spp. Pie charts illustrate the mean Q-values (genetic ancestry proportions estimated by STRUCTURE) across individuals at each site with the total number of individuals collected displayed above the pie chart. Inset map illustrates geographic context of Town Creek in the Tennessee River system of Alabama.

4. Discussion

The results of this study confirmed the presence of two non-native *Micropterus* spp. present in Town Creek, Alabama and demonstrated hybridization between the introduced black basses with native black basses. Hybrid fish were collected with total lengths ranging from 47 mm to 355 mm with some individuals possessing Q-values that are relatively low, while others are indicative of F1 hybrids (Table S1) providing evidence that hybridization is not merely a remnant of historical hybridization, but is actively ongoing. One possibility, deserving of further research, is that a source population of Alabama Bass exists in Lake Guntersville that may be actively migrating into Town Creek. There are no records from the Alabama Department of Conservation and Natural Resources (ADCNR) of any stocking of Alabama Bass or Coosa Bass into the Town Creek system in spite of their established presence. Interestingly, there are records of ADCNR stocking 9,000 fingerling-sized Smallmouth Bass into the High Falls Park area (Site 2) between 2007-2010 and another 3,000 fingerling-sized Smallmouth Bass at the Highway 227 bridge (Site 3) between 2007 and 2008 (Table S3). Despite stockings at two of the sample sites for this study, no pure Smallmouth Bass were captured by angling or electrofishing at any of the four sample sites. Introgressive hybridization of non-native Alabama Bass and Coosa Bass could be one explanation for the absence of Smallmouth Bass in this system. Another potential explanation is that Smallmouth Bass have been shown to migrate into lotic systems that experience seasonal water loss in the summer months (25). The present study was conducted during the months of July and August, and the Smallmouth Bass may have retreated to lower areas with more water, but further studies are needed to elucidate the potential role of seasonality on presence of Smallmouth Bass in Town Creek. Predation and/or angling may have also played a role in Smallmouth Bass elimination in the system.

Although it is unknown how and where Alabama Bass and Coosa Bass were initially introduced to Town Creek, the presence of Alabama Bass is especially concerning. Alabama Bass readily hybridize with other species of black bass, displacing native biodiversity (7, 8). In Norman Lake, NC, Alabama Bass have been shown to quickly transform the population structure of a predominately Largemouth Bass lake and become the most commonly caught black bass species (26). Although the oligotrophic conditions of Norman Lake favor the introduced Alabama Bass over the native Largemouth Bass, it is unknown what effect Alabama Bass can have in a eutrophic habitat like Lake Guntersville (27, 28). Lake Guntersville is a nationally-renowned Largemouth Bass fishery that generates over $4.6 million annually to the local economy in tournaments alone (29) with an overall value of $45.2 million (30). Additional surveys should be carried out to determine the extent of Alabama Bass invasion in the Lake Guntersville area and to quantify hybridization with native *Micropterus* spp.

Although angler introduction of Alabama Bass is well documented in southeastern reservoirs, there is far less evidence of Coosa Bass being stocked by anglers. This scenario seems unlikely in Town Creek, given that pure Coosa Bass were captured at most sites, with major geological barriers separating the upper three sites, such as a 40-foot waterfall (High Falls) at site two. One streamside resident mentioned that he has been catching Coosa Bass in Town Creek since at least 1980, indicating it is not a new introduction (Alvin Gilreath, personal communication). Another plausible explanation of Coosa Bass presence is through a historical headwater stream capture event between the Coosa and Tennessee rivers (31). The headwaters of Town Creek are in close proximity to the headwaters of Little River, a Coosa River tributary that contains native Coosa Bass. Notably, Town Creek is one of only two streams where the Rainbow Shiner (*Notropis chrosomus*) has been documented outside of its native Mobile Basin (32). The presence of two gamefish and one non-gamefish native to the Mobile Basin within Town Creek provides evidence for the potential of faunal exchange by way of headwater stream capture (31, 33, 34). Furthermore, geological evidence for such an event has been reviewed previously between the Coosa and Tennessee river systems (31). Finally, we note that the SNP markers utilized here (19), while capable of differentiating amongst the Alabama Redeye Bass complex, have lower diagnostic power in the Redeye Bass group than among other black bass species. While our STRUCTURE results, phenotypic characteristics, and geographic proximity, all strongly support their identification as Coosa Bass, further genetic marker development is ongoing which may serve to clarify the Redeye Bass complex in the near future.

The results of this study warrant more frequent monitoring of Alabama's streams and rivers using genetic tools. Hybrid individuals can present a large range of phenotypes that range from closely resembling one parent species to a mixed morphology of both parents, with backcrossing events further complicating proper field identification (35). A total of 62% of the fish collected for this study were mis-identified based on phenotype, almost double the error rate recorded in another recent study of black bass hybridization in the Chattahoochee River system (7). Results from phenotypic identification and subsequent genetic classification revealed that 100% of the discrepant individuals were hybrids misidentified as pure individuals. Pure Largemouth Bass were the only fish correctly identified 100% of the time by phenotype, while only a small number of hybrids (36%) were correctly identified as such in the field. The high error rate of correct field identification may be due, in large part, to the cryptic nature of phenotypes among the prevalent hybrids of Alabama Bass X Coosa Bass. Given that all individuals incorrectly identified by phenotype were, in fact, hybrids, the present study illustrates the need for genotyping in conjunction with field identifications to accurately assess populations and monitor for introductions and hybridization. Small-scale introductions in smaller streams, if revealed early, can be ameliorated through selective elimination of non-natives and/or restorative, supplemental stocking of natives (36). Conversely, and more commonly, failure to identify early hybridization events often allows for the eventual spread and dominance of introduced black basses and their hybrids.

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Chapter III Failure of Phenotypic Markers to Accurately Identify Black Bass Species and Associated Hybrids in Mobile River Basin, Alabama

Abstract

Hybridization among the black basses (*Micropterus* spp.) occurs due to weak postzygotic reproductive barriers and anthropogenic factors such as habitat alteration. Introduction of previously allopatric species can also result in hybrid swarms and in some cases, extirpation of native species. Introgressive hybridization is a major conservation concern for the more range-restricted black basses, which underscores the need for accurate identification of hybrids that often have cryptic phenotypes. To that end, we collected 1,723 fish from four river systems in the Mobile River Basin, Alabama, to compare phenotypic identification in the field based on morphology with genotype determined from single-nucleotide polymorphism (SNP) analysis using diagnostic markers for black basses. Results indicate that phenotypic identification among these cryptic hybrids is only 11% accurate. Only 4% of the fish collected were identified as hybrids in the field, whereas genotype analysis classified 22% of fish as hybrids. The majority of those misidentified in the field were hybrids between Alabama Bass (*M. henshalli*) and Redeye Bass (*M. coosae*) or those species along with another black bass species. Hybrid individuals composed 5-38% of the total number of fish collected from the four river systems, but 28-68% were misidentified in each river system. These results underscore the need for genetic tools to augment classic field surveys in the black basses as cryptic hybridization may lead to errant management decisions based on inaccurate species distribution assessments.

1. Introduction

Understanding the distribution of species is paramount to establishing native ranges and developing management and conservation plans. Black bass (*Micropterus* spp.) are one of the most popular freshwater sportfish and as such have been the subject of numerous sanctioned and illegal translocations (1). These introductions have resulted in numerous hybridization events that confound species distributions and identification (2-4). Although hybridization between the black basses does occur in nature, it is presumed to be a rare event (5, 6) unless congeners come into secondary contact due to anthropogenic introductions (7-10). This is especially concerning in the case of range-restricted black basses that are often geographically isolated from congeners and may be at risk if new black bass species are introduced (11).

One such group is the Redeye Bass *Micropterus coosae* complex in the Mobile River Basin (MRB), Alabama. Redeye Bass are the smallest black bass species, rarely reaching lengths of 305 mm total length. They mostly inhabit small to medium-sized streams with rocky substrate, generally above the Fall Line, an ecotone between the Piedmont and Coastal Plain physiographic provinces (12). Like many range-restricted black bass, Redeye Bass do not tolerate impoundment and are not found in reservoirs. These fish were recently proposed to be five unique species, each endemic to a particular drainage in the MRB and Chattahoochee River system (13). However, a lack of scientific consensus concerning the species status of this group exists due to the inclusion of hybrid individuals in species descriptions and uncertainty over clear diagnostic morphological and molecular characters (14, 15).

Redeye Bass are sympatric with Alabama Bass *Micropterus henshalli*, another black bass endemic to the MRB that grows faster and attains larger sizes than Redeye Bass (16). Unlike Redeye Bass, Alabama Bass are found throughout the MRB and thrive in reservoirs. Although introduced Alabama Bass frequently hybridize with native Redeye Bass outside of the MRB (10, 17), the extent of hybridization between these two native congeners in the MRB has not been studied. Accurate and reliable identification of pure and hybrid individuals is key to monitoring populations and developing conservation and management strategies. Although phenotypic identification is commonly used as a practical way to manage fish populations, hybrids and backcrossed individuals often display phenotypes that resemble one parent over the other (2, 4, 18, 19), underscoring the need for genetic tools to quickly and reliably determine species identity and to quantify hybridization. To date, only one study has examined the accuracy of phenotypic identification in hybridizing populations of Alabama Bass and Redeye Bass, and that study reported that 100% of hybrids had been mis-identified in the field (3).

Genetic markers such as microsatellites and single-nucleotide polymorphisms (SNPs) are often used to supplement morphological identification (8, 20). Although microsatellites have been the primary method used in numerous genetic studies of hybridization in fishes over the last few decades (8, 21-24), more recently SNPs have been widely adopted due to their cost-effectiveness and abundant distribution in both coding and noncoding regions of the genome (15, 25, 26). High-throughput, diagnostic markers such as SNPs are valuable in understanding hybridization and introgression in populations and can be used to inform short and long-term management decisions (15, 27, 28).

Redeye Bass are the subject of several ongoing studies to examine distribution and status of these species throughout the MRB. However, cryptic phenotypes among and within river systems appear to render field identifications of these fish questionable at best. Therefore, the objectives of this study were to document the accuracy of phenotypic identification of Alabama Bass, Redeye Bass, and their hybrids in the MRB using genetic identification with SNP markers diagnostic among the black basses (15). We hypothesized that due to the overlap of morphological features and complex hybridization that exists between these two species, phenotypic identification alone is insufficient to correctly identify these fishes in most cases.

2. Methods

2.1 Study Site

The MRB encompasses 114,000 square kilometers, including seven major river systems that comprise the largest Gulf Coast drainage east of the Mississippi River. These river systems drain 10 different physiographic provinces that each contribute unique physical and chemical characteristics to those waters. This has led to a variety of habitat types allowing the MRB to support some of the highest aquatic biodiversity in North America. Sympatric populations of Redeye Bass and Alabama Bass occur in the four largest basins in the MRB, the Black Warrior, Cahaba, Coosa, Tallapoosa rivers (29) (Figure 1). Each of these rivers originate in upland physiographic provinces and flow southward over the Fall Line into the Coastal Plain Province. Streams and rivers sections upstream of the Coastal Plain in each basin are generally characterized by rocky substrate, high gradient, and moderate to high water velocities; whereas those in the Coastal Plain tend to be low gradient and highly sinuous with sandy bottoms and little to no rocky substrate (Boschung and Mayden 2004). The mainstems of the Tallapoosa and Coosa rivers are fragmented by numerous hydropower dams; whereas the Cahaba River has only a few low-head dams in the vicinity of Birmingham, Alabama, (30, 31). Likewise, the Black Warrior mainstem above the Fall Line is mostly impounded. However, most of the tributary streams in all four basins are free-flowing, although with varying levels of disturbed land uses such as urban and agriculture (Boschung and Mayden 2004).

2.2 Sample Collection

Each of the four watersheds were sampled for this study. Sample sites with potential hybridization were selected based on previous collection data (13), angler reports, and visual assessment of stream characteristics. Sampling occurred from March 2018 through August 2020 at 95 different streams throughout the MRB (Figure 1). Black bass were collected by both angling and electrofishing due to different study designs, with angling targeting putative pure Redeye Bass in specific streams and electrofishing employed to investigate distribution and hybridization. Only putative Redeye Bass were collected in the angling samples; whereas, all black basses were collected by electrofishing. Fish were measured to the nearest total length (TL) (mm), weighed (g), and phenotypically identified to species using major distinguishing characters (29) and recorded as Alabama Bass (*M. henshalli*, Largemouth Bass (*M. salmoides*), Redeye Bass (*M. coosae*), or hybrid (Table S1). Redeye Bass were identified from all other black basses by the presence of a silver to blue crescent on the upper posterior half of the eye, white edges on the superior and inferior margins of the caudal fin, and the lack of coalescence in long vertical blotches along the lateral line. Largemouth Bass *M. salmoides* were identified based on absence of dorsolateral quadrate markings and a jaw that extends past the eye when closed. Alabama Bass were identified by the presence of a tooth patch on the tongue, horizontal rows of spots below the lateral line, and a jaw that does not extend beyond the eye when closed. Individuals that met none of these criteria or that had features of more than one species were assumed to be hybrids since no other black basses are documented to occur in these systems. All individuals were photographed for phenotypic record, and fin clipped for genetic analysis. Fin clips were placed into 95% ethanol (EtOH) for storage and transport.

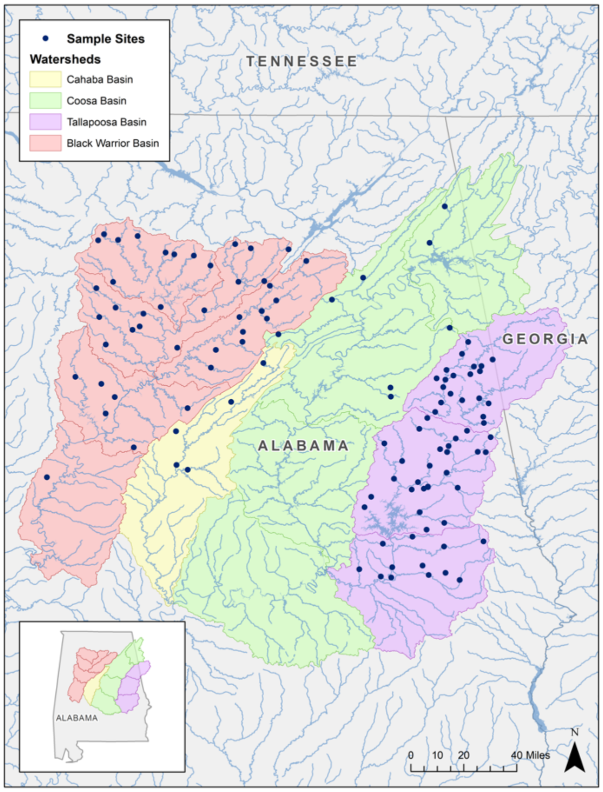


Fig. 1 Sample sites within the Mobile River Basin (MRB) that were sampled in 2018 through 2020 by both angling and electrofishing. The river basins are illustrated by different colors and sample sites are indicated by dots.

2.3 SNP Genotyping

Genomic DNA was extracted using a simple sodium hydroxide (NaOH) and hydrochloric acid (HCl) tissue digestion (32). Genomic DNA was quantified using the Nanodrop 2000c spectrophotometer (ThermoFisher, Waltham, MA, USA). Samples were genotyped using a 64-SNP panel designed previously from genotype-by-sequencing (GBS) data for classification across six to nine *Micropterus* species (15). Amplified and mass-specific extension reactions (iPLEX™ Gold Assay) were performed using 2 µL of input DNA at concentrations ranging from 34-351 ng/µL (33). Genotypes were generated using the MassARRAY (Ageno Bioscience Inc., San Diego, CA) system according to manufacturer's protocols. SNP genotyping calls were generated automatically using the MassARRAY Typer 4.0 Analysis software. All samples were processed with a positive control of Smallmouth Bass DNA with known genotype and a total of 20 individuals were run on multiple plates with 99.6% of genotypes matching among replicates (data not shown).

2.4 Data Analysis

Genotype data were analyzed to identify species and hybridization status of the *Micropterus* samples from the MRB using a Bayesian clustering algorithm-based program, STRUCTURE version 2.3.4 (34). The STRUCTURE runs were performed assuming six populations (K=6), as six reference *Micropterus* species' genotypes were included in each analysis (Alabama Bass (*N*=70), Largemouth Bass (*N*=70), Redeye Bass (*N*=60), Shoal Bass *M. cataractae* (*N*=70), Smallmouth Bass *M. dolomeiu* (*N*=70), and Spotted Bass *M. punctulatus* (*N*=70)). Thongda et al. (2019) optimized the 64-plex black bass panel for use with these six species. Individual STRUCTURE runs were performed with previously collected reference individuals representative of the Redeye Bass population in the drainage where that specific collection was made (15). Shoal Bass were chosen as a reference species over other black basses due to their geographical proximity to the sample sites following (3). STRUCTURE analyses were performed using the admixture model (K=6) with correlated allele frequencies in conjunction with the USEPOPINFO and POPFLAG models with a migration prior set to 0.01 and a burn-in of 20,000 iterations followed by 200,000 repetitions of Markov chain Monte Carlo (MCMC) simulation (35). STRUCTURE runs were performed in triplicate using the R package *strataG* (36) with individual ancestry proportion values (Q-values) averaged across replicates.

STRUCTURE results with individual Q-values of ≥0.95 were assigned to a single species (i.e., "pure"). For hybrid individuals, a Q-value threshold of ≥0.05 for a species was required to be considered a contributing ancestry proportion (37). Correct phenotypic identification rates were calculated for each species and compared between rivers and among different size classes of each species using the Fisher Exact Test (38) as implemented in the R package *RVAideMemoire* (v. 0.9-79), with Pvalues adjusted to control the false discovery rate (39).

3. RESULTS

3.1 Sample Collection and Phenotypic Identifications

In total, 1,723 black bass specimens were collected in this study, 530 by angling and 1,193 by electrofishing. Fish collected by river system ranged from 138 in the Cahaba drainage to 803 in the Black Warrior drainage (Table 1). Fish ranged in length from 27 mm to 503 mm TL, with a mean of 193 mm (SD = 76 mm). Redeye Bass and Alabama Bass were collected in all four basins, but Largemouth Bass were only collected in the Tallapoosa and Black Warrior river basins. Likewise, hybrid individuals were only identified in the Tallapoosa and Black Warrior river basins; however, very few individuals were identified as hybrids in the field (Table 1).

Table 1. Number of black bass collected and field identified to species in four river basins within the Mobile River Basin of Alabama for genetic examination. Number in parentheses is the percent of field identifications that were determined to be incorrect for each putative species or hybrid using genotyping.

Species	Tallapoosa	Coosa	Cahaba	Black Warrior	All
Alabama	138 (33.3)	12 (12.7)	10 (0)	402 (48.8)	562 (43.4)
Redeye	425 (4.9)	141 (4.3)	128 (22.7)	264 (33.3)	958 (15.0)

Largemouth	47 (2.1)	0 (-)	0 (-)	84 (6.0)	131 (4.6)
Hybrids	19 (68.4)	0 (-)	0 (-)	53 (28.3)	72 (38.9)
Totals	629	153	138	803	1,156

3.2 SNP Population Genetics Analysis

Formal assignment of ancestry proportions at K=6 of the 1,723 individual black bass specimens from four different watersheds in the Mobile Basin in Alabama confirmed hybridization among five different black bass taxa, including native Alabama Bass, Redeye Bass, and Largemouth Bass and non-native Spotted Bass and Smallmouth Bass (Figures 2 and 3; Table 1; Tables S1 and S2). The majority of fish collected were Redeye Bass (*n*=823), followed by Alabama Bass (*n*=340), and their hybrids (n=382) with smaller numbers of Largemouth Bass (*n*=176) and Smallmouth Bass (*n*=2).

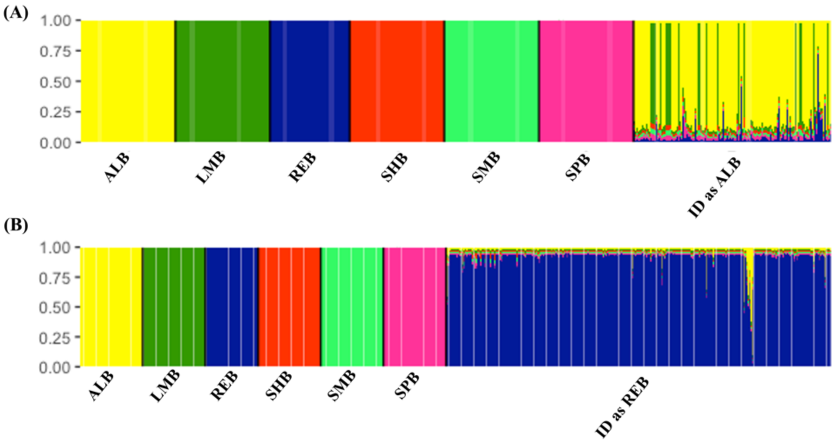


Fig. 2 Taxonomic assignments in STRUCTURE of individuals collected from the Tallapoosa River drainage identified in the field as (A) Alabama Bass (ALB) and (B) Redeye Bass (REB). Figure 2A shows there was variable levels of admixture and that some LMB were identified as

ALB in the field. Figure 2B shows identification of REB in the field was mostly accurate, but there were some hybrid individuals that were cryptic. Distinct clusters are illustrated by different colors, with each individual's proportional assignment to those clusters represented by vertical bars.

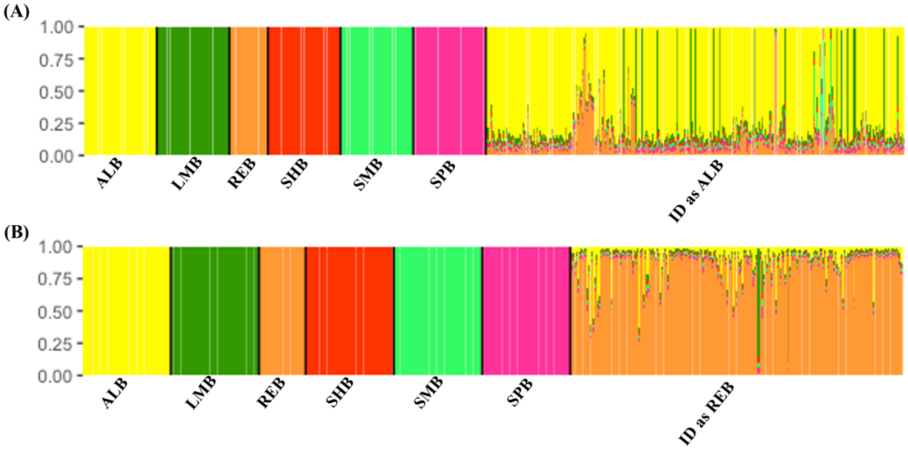
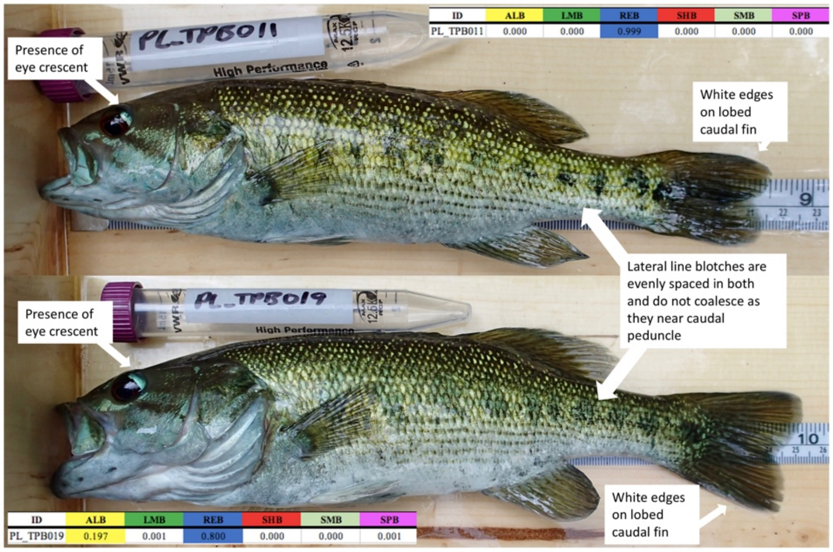


Fig. 3 Taxonomic assignments in STRUCTURE of individuals collected from the Black Warrior River drainage identified in the field as (A) Alabama Bass (ALB) and (B) Redeye Bass (REB). Figure 3A shows that some LMB were incorrectly identified as ALB in the field. Both Figures 3A & 3B show that many of the individuals identified as ALB or REB in the field were actually hybrids of the two, or additional black bass combinations.

Genotype analysis revealed the largest error in field identification relative to genotype identification was among the hybrid individuals. Almost 40% of individuals field identified as hybrids were in fact pure fish (Table 1). This was especially evident in the Tallapoosa River basin, where almost 70% of putative hybrids were actually pure fish. Overall, only 4% of the fish collected were identified as hybrids in the field, while genotype analysis indicated that 22% of fish collected were hybrids (Table S1). Of those, 85% were hybrids between Alabama Bass and Redeye Bass or Alabama Bass, Redeye Bass, and another black bass species (Table 1). Alabama Bass X Spotted Bass hybrids composed 10% of the hybrids sampled, with 93% of those incorrectly identified as Alabama Bass (Table S1).

Focusing on the systems with the two larger sample sizes, STRUCTURE bar plots illustrate that many fish identified in the field as Alabama Bass or Redeye Bass from the Tallapoosa River system (Figure 2) and the Black Warrior River system (Figure 3) were actually hybrids with varying degrees of admixture. Phenotypic identification of Redeye Bass was more reliable in the Tallapoosa River basin (5% error) compared to the Black Warrior River system, where a third of the fish identified as Redeye bass were in fact hybrids (Table 1; $P < 3.4e-22$). Alabama Bass phenotypic identification had higher error rates than Redeye Bass in the Tallapoosa River system (33% vs 5%) and the Black Warrior River (49% vs 33%) system ($P < 0.006$). In both systems, Largemouth Bass were misidentified as Alabama Bass (Figures 2 and 3), but the amount of admixture was more pronounced in the Black Warrior River system (Figure 3) than the Tallapoosa River system (Figure 2). Error rates in phenotypic identification of hybrids involving Redeye Bass and Alabama Bass varied between the Tallapoosa (68%) and Black Warrior (28%) drainages ($P < 0.03$) (Table 1;Table S1). Although the Coosa and Cahaba rivers had smaller sample sizes than the Black Warrior and Tallapoosa rivers, the error in reliable field identification of hybrids remained high (100%) for both, with the majority of those consisting of hybrids between Alabama Bass and Redeye Bass (Table S1). The incidence of hybridization found in the Cahaba basin was higher than that in the Coosa basin, despite similar sample sizes (Table S1).

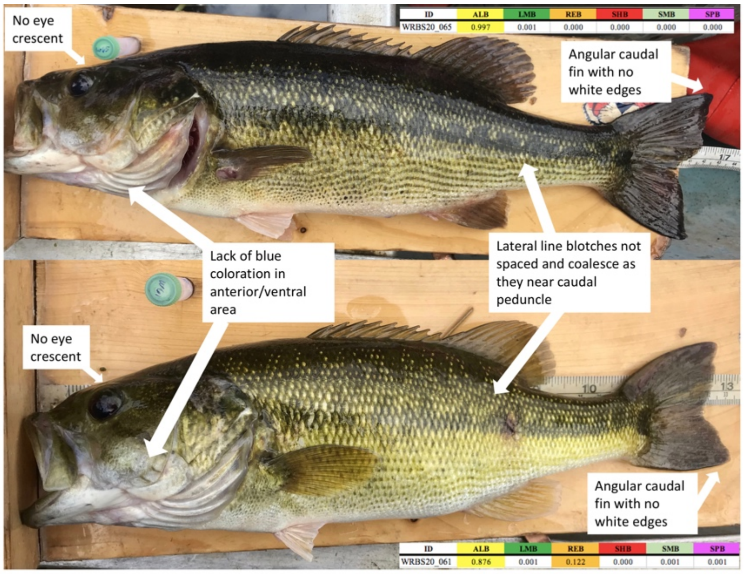
Accurate field identification was especially problematic in hybrids between Redeye Bass and Alabama Bass, and among other black basses, as they were accurately identified in the field only 11% of the time (Table 1). The morphology among Redeye Bass X Alabama Bass hybrids results in a cryptic phenotype that precludes accurate and reliable field identification. Photographs taken during field identification illustrate the characteristics of genetically confirmed Redeye Bass, Alabama Bass, and their hybrids (Figures 4-6). In the Tallapoosa River system, morphological and color differences previously reported to be diagnostic for Redeye Bass were present in hybrid individuals that resembled pure Redeye Bass with a lack of blotches that coalesce along the lateral line, blue eye crescent on posterior upper half of eye, and lobe-shaped caudal fins with white edges on the superior and inferior margins (Figure 4). Similarly, fish that phenotypically resembled Alabama Bass by lacking the eye crescent, white edges on caudal fin, and any coloration in the fins indicative of Redeye Bass were confirmed by genotyping to be a hybrid between Alabama Bass and Redeye Bass (Figure 5).



ID	ALB	LMB	REB	SHB	SMB	SPB
PL_TPB011	0.000	0.000	0.999	0.000	0.000	0.000

ID	ALB	LMB	REB	SHB	SMB	SPB
PL_TPB019	0.197	0.001	0.800	0.000	0.000	0.001

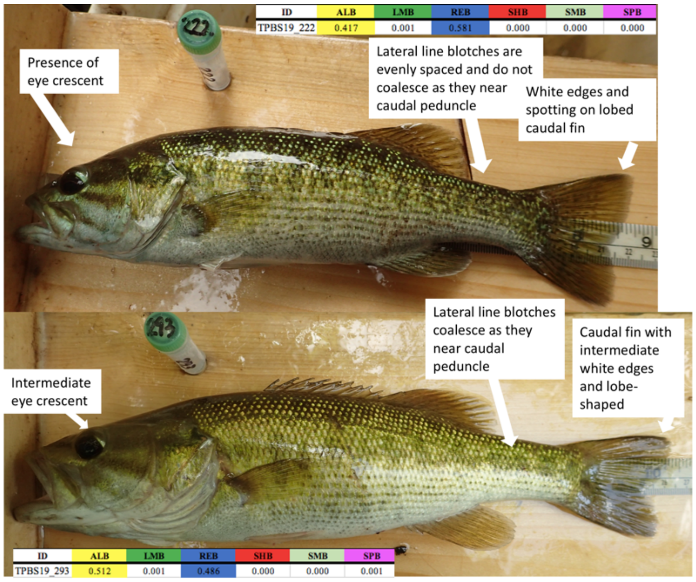
Fig. 4 Photograph from field sampling illustrate morphological characteristics to identify Redeye Bass from other black bass species cannot differentiate between a pure individual (top) and hybrid individual (bottom) from the Tallapoosa River drainage. Both pure and hybrid individuals have characteristics of REB: eye crescent, vertical blotches that do not coalesce along the lateral line, and lobe-shaped caudal fins with superior and inferior white edges indicating the cryptic nature of ALB X REB hybrids.



ID	ALB	LMB	REB	SHB	SMB	SPB
WRBS20_065	0.997	0.001	0.000	0.000	0.000	0.000

ID	ALB	LMB	REB	SHB	SMB	SPB
WRBS20_061	0.876	0.001	0.122	0.000	0.001	0.001

Fig. 5 Photograph from field sampling illustrate morphological characteristics to identify Alabama Bass from other black bass species cannot differentiate between a pure individual (top) and hybrid individual (bottom). Both pure and hybrid individuals have characteristics of ALB: no eye crescent, vertical blotches coalesce along lateral line, and angular caudal fin with no white edges indicating the cryptic nature of ALB X REB hybrids.



ID	ALB	LMB	REB	SHB	SMB	SPB
TPBS19_222	0.417	0.001	0.581	0.000	0.000	0.000

ID	ALB	LMB	REB	SHB	SMB	SPB
TPBS19_293	0.512	0.001	0.486	0.000	0.000	0.001

Fig. 6 Photograph from field sampling illustrate the varied morphological characteristics in hybrids with equal genomic ancestry (top and bottom) can favor one parent over the other. One hybrid (top) contains characteristics of a pure REB: eye crescent, vertical blotches separated along total length of the lateral line, white edges on inferior and superior margins of caudal fin.

The other (bottom) has intermediate features of both parents: eye crescent, vertical blotches do coalesce along the length of the lateral line, and intermediate white edges on superior and inferior margins of caudal fins.

Identification error rate of Redeye Bass X Alabama Bass hybrids was lowest at ancestry proportions (Q-value) ranges between 0.401-0.600, however, incorrect identification was still over 40% in this range (Figure S1). Phenotype was also variable among fish with equal genomic contributions from Alabama Bass and Redeye Bass. Some individuals possessed intermediate phenotypes of both parent species such as presence of an eye crescent with blotches along the lateral line that coalesce as they near the caudal peduncle. In other cases, the hybrids with equal genomic contributions from both parents had the morphology of only one parent (Figure 6).

Size of collected fish did not have a strong, consistent effect on accuracy of phenotypic field identification (Table S3). Redeye Bass with total lengths up to 300 mm were identified correctly over 80% of the time in the field. In contrast, Alabama Bass, regardless of total length were identified correctly ranging from 43% to 67% of the time. Interestingly, the largest size class of both Alabama Bass and Redeye Bass had the highest error rates (Table S3).

4. Discussion

The results of this study confirm that Redeye Bass, Alabama Bass, or complex hybrids involving multiple black basses, are often misidentified in the field based on phenotypic characteristics. Correct phenotypic identification in the field is often a crucial first step for state agencies charged with managing natural resources in order to evaluate distribution and other metrics relevant to a population of interest. Our data show that a dependence on phenotypic identification in streams where Redeye Bass and Alabama Bass occupy similar reaches would allow many hybrid populations to remain undetected.

Redeye Bass were correctly identified 95% of the time based on phenotype in the Tallapoosa River system compared to 67% in the Black Warrior River system. A similar trend was observed comparing phenotypic identification of putative Redeye Bass between the Coosa and Cahaba river systems. Conversely, field identification of Alabama Bass based on phenotype was less accurate with 67% identified correctly in the Tallapoosa River system compared to 51% in the Black Warrior River system. Differences between Alabama Bass and Redeye Bass accuracy rates are likely due to sample site selection being biased toward native Redeye Bass habitat. Differences in accuracy of Alabama Bass field identification between rivers are likely due to the differing frequencies of hybrids collected between drainages. Our data indicate that hybridization is higher in the Black Warrior and Cahaba drainages relative to the Tallapoosa and Coosa drainages, although due to the difference in collection methods and study designs, the only two drainages we can directly compare are the Tallapoosa with the Black Warrior, and the Coosa with the Cahaba.

The largest error in field identification relative to genotype identification was among hybrid individuals. Only 4% of the fish collected were identified as hybrids in the field, while genotype analysis confirmed 22% of fish collected were hybrids. Of those hybrids misidentified, 83% were hybrids between Alabama Bass and Redeye Bass. The error rates of phenotypic identification of hybrids in all four systems were high. In the Coosa and Cahaba systems, no fish were identified in the field as hybrids, despite there being 13% hybrids based on genotyping in those systems. Of those identified in the field as hybrids, 68% were identified correctly in the Tallapoosa River and 28% in the Black Warrior River.

Redeye Bass and Alabama Bass are phenotypically differentiated based on meristic attributes and morphological differences such as scale counts, fin coloration, and vertical blotch patterns along the lateral line. Although meristic counts are limited in their ability to detect hybridization (40), morphological characteristics for these fish have been reported previously (13, 41).

Redeye Bass should be diagnosable from all other black basses based on a silver to blue crescent on the upper posterior half of the eye, white edges on the superior and inferior margins of the caudal fin, and the lack of coalescence in long vertical blotches along the lateral line (13). Alabama Bass have blotches along their lateral line that differ from Redeye Bass through the presence of more numerous blotches that are wider than the vertical length and coalesce as they near the caudal peduncle. Intermediate forms of these characteristics have been observed in hybrids of Redeye Bass and Alabama Bass (13, 14, 41). Unfortunately, there is considerable intraspecific variation and interspecific overlap in these characteristics among these two congeners (13, 41) making phenotypic identification questionable and fraught with error. These similarities can be further amplified in later generation crosses and backcrossed individuals. In addition, lateral line blotches can be affected by stress and ambient temperature leading to differences in interpretation based on a fish's individual stress response and ambient conditions (4, 13). These cryptic phenotypes underscore the need for genetic tools to augment surveys of populations composed of Redeye Bass and Alabama Bass.

Previous studies have shown that F1 hybrids are easier to identify based on intermediate characteristics of both parents (2, 4), with the ability to detect hybridization deteriorating with increasing backcrossing (19, 42). However, our data suggest that while Alabama Bass X Redeye Bass with equal ancestry proportions are more likely to be correctly identified in the field than complex hybrids, they are still often cryptic with attributes of one parent masking those of the other. If a single parental phenotype dominates, a complete reliance on phenotypic identification can obscure evidence that hybridization is occurring and may lead to errant management decisions in these range-restricted populations of black bass.

Total length of fish played a role in proper field identification of some species, but generally improved with increasing total length. This trend is logical due to the fact that juvenile centrarchids are known to be difficult to discern due to less developed coloration patterns than those used to identify adults (43). Alabama Bass were commonly misidentified (43-67%) regardless of size class. Redeye Bass were correctly identified (80-88%) more often than Alabama Bass, although above 301 mm TL, correct field identification of Redeye Bass dropped sharply from over 80% to 36%. This could be due to larger Redeye Bass possessing characteristics not commonly observed. Hybrids had the lowest percentage of correct field identification based on size, which was expected given the overall inability to diagnose hybrids in the field. The phenotype observed from the collection images correspond to the field identification, indicating that the phenotype is more cryptic in hybrids.

To our knowledge, this is the first data that document and quantify the inaccuracy of phenotypic identification between these two sport fishes in a system where they have evolved in sympatry. The results of this study also emphasize the need to re-visit the species descriptions of the Redeye Bass in the MRB based on the documented high levels of cryptic hybridization in some streams (14, 15). These data should be useful to fisheries managers and biologists in their efforts to study and conserve this important group. A potential limitation of this dataset is the subjective identification of individuals in the field, particularly due to the lack of previous knowledge of hybrid phenotypes. However, observer bias was limited by education and field training on the general diagnostic features of each species and their hybrids in accordance with the only published paper that identified a few intermediate characteristics for identifying hybrids of Alabama Bass and Redeye Bass from the Black Warrior River (13). Retrospectively, there appear to be no consistent morphological characteristics with which to identify hybrids.

This study investigated the accuracy of phenotypically identified hybrids between Alabama Bass and Redeye Bass in the Mobile Basin when compared to genotypic identification. Our results indicate that hybrids between Redeye Bass and Alabama Bass are cryptic leading to unreliable identification when relying on phenotypic characteristics in the field. In order to investigate hybridization and population dynamics in these systems, genotyping will be required to supplement existing morphological identification. Ongoing efforts in our laboratory are aimed at generating finer-scale genotype-based surveys to better identify and classify pure and hybridizing populations, highlight trends in introgression, and delineate environmental factors contributing to hybridization in these diverse systems.

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Chapter IV Survey of Hybridization Between Sympatric Redeye Bass (*Micropterus coosae*) and Alabama Bass (*M. henshalli*) in Mobile River Basin, Alabama

Abstract

Hybridization by introduced taxa is one of the main threats to the diversity among the black basses (*Micropterus* spp.) and particularly relevant to the Redeye Bass (*M. coosae*, REB). The Mobile River Basin in Alabama contains one of the largest populations of native Redeye Bass; however, little is known about the levels of hybridization between sympatric Redeye Bass and Alabama Bass (*M. henshalli*, ALB) in this system. We used 64 diagnostic SNP loci to determine the extent of hybridization in 2,679 fish collected from 135 sites across three major river systems. About 28% (750/2,679) were identified as hybrids, 42% (1,135/2,679) were identified as pure REB, 21% (552/2,679) were identified as pure ALB, and the remaining individuals were identified as pure Largemouth (240/2,679) and Smallmouth Bass (2/2,679). We found evidence of hybridization at 54% of the sites (73/135) with only 46% of sites (62/135) having no evidence of hybridization. We found that hybridization rates varied among river drainages and streams within those drainages. The Black Warrior River drainage contained the highest levels of overall hybridization (34%). The Cahaba River drainage had an overall hybridization rate of 31% followed by the Tallapoosa River (13%). Based on our sampling, individual streams ranged in hybridization rates from 0-75%. The majority of hybrids (76%) were between REB and ALB, and primarily consisted of backcrossed individuals. This indicates a bimodal hybrid zone indicative of a hybrid swarm in these two sympatric populations. Our results support the hypothesis that hybridization with sympatric ALB is occurring at very high

levels across the native range of REB and identifies genetically pure Redeye Bass populations that should be prioritized for conservation. Introgressive hybridization is occurring at dynamic levels in these co-evolved species that equal and in some cases, exceed hybridization rates between previously allopatric black bass populations. This introgressive hybridization threatens the local biodiversity of these range-restricted REB populations across the heart of their native range.

1. Introduction

The Mobile River Basin (MRB) ranks third in species richness for North America, making it one of the most speciose assemblages of freshwater fishes (1). The black basses (*Micropterus* spp.) are some of the most ecologically and economically important freshwater gamefish in the United States (2). The popularity of black bass among anglers has led to widespread translocations to create and enhance fishing opportunity (3). Black bass rely on external fertilization and their weak reproductive barriers render them susceptible to hybridization events even among divergent taxa (4, 5). Although natural hybridization is an important aspect of gene flow that allow populations to evolve by serving as a source of novel genotypes and epistatic interactions to adapt to changing conditions (6), human-mediated hybridization has resulted when previously isolated taxa are brought together (7-9). Hybridization can result in hybrid offspring that are less fit than parental forms and selected against, but if hybrid offspring survive and are able to contribute alleles to future generations, it is termed introgressive hybridization (10). Introgressive hybridization between native and introduced species can lead to the creation of hybrid swarms, bimodal hybrid zones, and complete replacement of native species that threaten the biodiversity among the black basses (*Micropterus* spp.) (9, 11-15). Hybrid zones occur when two genetically divergent populations come in contact and interbreed (16). Hybrid zone size and structure can be influenced by hybrid dispersal, abundance of parental types, and reproductive isolating mechanisms (5, 17). Therefore, effective management of hybridized populations must be guided by the factors that facilitate hybridization as well as the frequency and spatial scale of hybridization.

Recent technological advances have made genomic approaches more widespread to investigate the diversity within the *Micropterus* genus (18), which is composed of 19 different species (19). Many of these species have restricted native ranges that make them susceptible to ecological threats and threatening the long-term viability of their unique genetic lineages (18, 20, 21). Of these, the Redeye Bass (*M. coosae*; REB) are endemic to the stream systems above the Fall Line in Alabama, Georgia, South Carolina, and Tennessee in the Mobile River Basin (MRB) (22, 23). Originally described in 1940 (24), REB were recently split into five species (22) with two more awaiting formal description (23). Despite concerns of proper taxonomy, these described and proposed species do have significant phylogenetic support based on a recent study utilizing ddRAD loci indicating monophyly of the REB species complex (19). Alabama Bass (*M. henshalli*; ALB) occur in sympatry with REB in the MRB, but differ in their habitat preferences (22, 25). REB are fluvial specialists that prefer the cooler water and steeper gradient of smaller upland streams (26-28) while ALB are more of a species generalist that inhabit both lentic and lotic systems (24, 25, 29, 30). When found in overlapping stream reaches, REB typically occupy different niches than ALB, indicative of some form of habitat partitioning (27).

While the majority of hybridization studies in the black basses focus on the introduction of non-native species resulting in widespread hybridization and potential for extirpation and extinction (8, 11, 12, 15, 31, 32), very little is known about hybridization among between species that naturally occur in sympatry. Natural hybridization is thought to be rare among the black basses (20, 33), but can occur in fishes by parental species with disparate abundances, external fertilization, and weak reproductive isolating mechanisms (4, 34). Hybrids between REB and ALB have been observed in the MRB (22, 24, 25) and previous studies have shown that ALB and REB are sister taxa (19, 21, 23), owing their relationship to historical hybridization between the two overlapping species (19, 21). Although these sister taxa have unique evolutionary lineages (19), much work is still needed to elucidate why these two species hybridize so readily.

Anthropogenic activities such as agriculture, pollution, urbanization, altered temperature regimes, and mining on a landscape scale can promote hybridization by weakening reproductive barriers through increased turbidity (35, 36) and affect the ecological integrity of a stream by reducing stream heterogeneity (36). Human-mediated disturbances such as translocations and habitat fragmentation can exacerbate and compound these factors and facilitate hybridization between native species (37, 38). This human-mediated hybridization can have deleterious effects on local biodiversity by causing erosion of native genomes, outbreeding depression, genetic swamping, and extinction (5, 14) and is considered to be one of the primary threats to freshwater ecosystems (39). Streams impacted by anthropogenic disturbances have been shown to have shifted fish assemblages to more tolerant and generalist fishes with overall lower diversity and abundance relative to undisturbed streams (36, 40).

Recently single-nucleotide polymorphisms (SNPs) have become more preferred to investigate hybridization due to their abundance across the genome, reproducibility, and their ability to be automated (41-43). In this study, we use 64 diagnostic SNPs (44) to survey and quantify the levels of admixture between sympatric REB and ALB across three major river systems (Tallapoosa, Black Warrior, Cahaba) in the MRB of Alabama. Field identification has been shown to be unreliable in population surveys between these sister taxa due to cryptic phenotypes resulting from introgressive hybridization (45). Therefore, genetic surveys are crucial to identify pure populations of REB and quantifying the extent of hybridization with sympatric ALB. This assessment will establish a baseline of where hybridizing populations occur allowing for future investigation into the factors that may facilitate hybridization and inform the development of conservation and management strategies.

2. Methods

2.1 Study Site

The MRB encompasses 114,000 square kilometers, including seven major river systems that comprise the largest Gulf Coast drainage east of the Mississippi River. These river systems occur in 10 different physiographic provinces allowing for some of the highest aquatic biodiversity in North America. Sympatric populations of REB and ALB occur in the four largest basins in the MRB, the Black Warrior, Cahaba, Coosa, Tallapoosa rivers (46) (Figure 1). Each of these rivers originate in upland physiographic provinces (Piedmont and Cumberland Plateau) and flow southward over the Fall Line into the Coastal Plain Province. Upland streams are generally characterized by rocky substrate, high gradient, and moderate to high water velocities. Rivers and streams in the Coastal Plain tend to be low gradient and highly sinuous with sandy bottoms and little to no rocky substrate (Boschung and Mayden 2004). The mainstems of the Tallapoosa and Black Warrior rivers are fragmented by numerous hydropower dams. In contrast, the Cahaba River has only a few low-head dams in the vicinity of Birmingham, Alabama, (47, 48). Most of the tributary streams in all four basins are free-flowing, although each have varying levels of disturbed land uses such as urban and agriculture (Boschung and Mayden 2004).

2.2 Sample Collection

Each of the four watersheds were sampled for this study. Sample sites with putative REB populations were selected based on previous collection data (22), angler reports, and visual assessment of stream characteristics. Sampling occurred during the spring and summer months from March 2018 through November 2021 at 135 different streams throughout the MRB (Figure 1). Most streams were only sampled once during this period, although five streams were sampled at least twice. Mean stream width (MSW) was determined by haphazardly measuring five to seven widths within the first 50 M of the study site with a handheld laser rangefinder. MSW was multiplied by 90 seconds and divided by 60 to obtain the timed transect length in minutes for each site. Transects were separated by a minimum of 10 MSW to ensure there was no immigration of bass from previously sampled sites. Site and transect-level covariates were collected to determine factors associated with hybrid occurrence and hybrid counts. Bank stability and vegetative cover were assessed visually and scored according to the Georgia Stream Team Protocol (49). All scores were averaged across each transect to determine the overall score for each site.

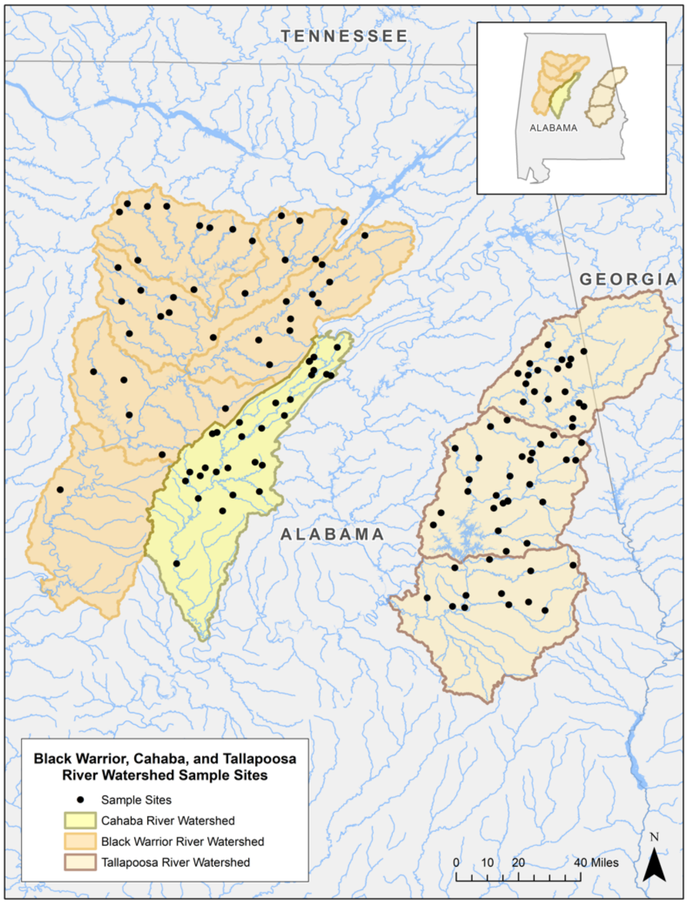

Fig. 1 Streams sampled in 2018 through 2020 within the Mobile River Basin (MRB) are indicated by dots. The river basins are illustrated by different colors.

The sampling gear used was a DC electrofishing unit powered by a 2000-Watt Honda generator and a hand-held anode operated out of a 4.6-m aluminum canoe (50). The anode was a 3 m pole with a 35 cm ring with a mesh net. Transects were sampled in a downstream direction and alternated between left and right banks to target all habitat. Streams not suitable for canoe shocking (<8 MSW) were sampled with one or two Smith Root LR-24 backpack units. Backpack streams were sampled in an upstream direction over a standard distance of 40 MSW, measured as described above.

Turkey and Hubbard creeks in the Black Warrior River drainage were sampled by angling due to the presence of the federally-listed Black Warrior waterdog (*Necturus alabamensis*) which precluded electrofishing those reaches. Regardless of collection method, all black bass species were captured, measured to the nearest total length (TL) (mm), weighed (g), phenotypically identified to species using major distinguishing characters (46), and recorded as ALB, REB, Largemouth Bass (*M. salmoides*; LMB), or hybrid (Table S1). All individuals were photographed for phenotypic record, and fin clipped for genetic analysis. Fin clips were placed into numbered tubes containing 95% ethanol (EtOH) corresponding to an individual fish for preservation and genetic analyses.

2.3 SNP Genotyping

Genomic DNA was extracted using a simple sodium hydroxide (NaOH) and hydrochloric acid (HCl) tissue digestion (51). Genomic DNA was quantified using the Nanodrop 2000c spectrophotometer (ThermoFisher, Waltham, MA, USA). Samples were genotyped using a 64-SNP panel designed and optimized previously from genotype-by-sequencing (GBS) data for classification across six to nine *Micropterus* species (44). Amplified and mass-specific extension reactions (iPLEX™ Gold Assay) were performed using 2 µL of input DNA at concentrations ranging from 34-351 ng/µL (52). Genotypes were generated using the MassARRAY (Ageno Bioscience Inc., San Diego, CA) system according to manufacturer's protocols. SNP genotyping calls were generated automatically using the MassARRAY Typer 4.0 Analysis software. All samples were processed with a positive control of Smallmouth Bass DNA with known genotype and a total of 20 individuals were run on multiple plates with 99.6% of genotypes matching among replicates (data not shown). Genotype data was converted to a STRUCTURE input file using PGDSpider version 2.1.1.5 (53).

2.4 Data Analysis

Genotype data were analyzed to identify species and hybridization status of the *Micropterus* samples from the MRB using the Bayesian clustering algorithm implemented in STRUCTURE version 2.3.4 (54). The STRUCTURE runs (K=6) were performed with genotypes from six reference *Micropterus* species included in each analysis (Alabama Bass (*N*=70), Largemouth Bass (*N*=70), Redeye Bass (*N*=60), Shoal Bass *M. cataractae* (*N*=70), Smallmouth Bass *M. dolomeiu* (*N*=70), and Spotted Bass *M. punctulatus* (*N*=70)). Individual STRUCTURE runs were performed with previously collected reference individuals representative of the Redeye Bass population in the drainage where that specific collection was made (44). All reference taxa were chosen over other black basses due to their geographical proximity to the sample sites following (55). STRUCTURE analyses were performed using the admixture model with correlated allele frequencies in conjunction with the USEPOPINFO and POPFLAG options with a migration prior set to 0.01 and a burn-in of 20,000 iterations followed by 200,000 repetitions of Markov chain Monte Carlo (MCMC) simulation (56). STRUCTURE runs were performed in triplicate using the R package *strataG* (57) with individual ancestry proportion values (Q-values) averaged across replicates. STRUCTURE results with individual Q-values of ≥0.95 were assigned to a single species (i.e., “pure”). For hybrid individuals, a Q-value threshold of ≥0.05 for a species was required to be considered a contributing ancestry proportion (58). Results from a subset of these samples have been previously published to compare phenotypic accuracy of field identification (45), while this paper focuses on the distribution of pure and hybridized populations.

2.5 Data Extraction from GIS

Adapting previously published methods (59), we used ArcGIS Pro 2.9.0 to extract independent variables for each transect from the National Hydrography Dataset (NHD) Plus Version 2 (60) and the Comprehensive Southeast Aquatic Barrier Inventory (CSABI) (61). The NHD is broken down into interconfluence stream segments (62). For each individual stream segment, the NHD provides data at two scales: the local catchment (i.e., only the land that drains directly into the segment) and the watershed (the cumulative total watershed for the segment and all upstream segments).

We plotted each transect as a point in ArcGIS Pro and used the Spatial Join tool to match each transect point to its corresponding stream segment and data. For each transect, we collected data about the area of the local catchment and the watershed; the maximum elevation, minimum elevation, and slope of the local catchment; and the land cover proportions within the local catchment and the watershed (Table S4). The area, elevation, and slope data are included as attribute tables within the NHD. The land cover data is part of an NHD supplementary dataset (63) that compiles 2016 National Land Cover Database (NLCD) land cover data for each segment of the NHD. The local catchments and watersheds for our transect sites included 15 NLCD land cover classes, which we reclassified into the following five categories: natural vegetation (deciduous forest, evergreen forest, mixed forest, shrub/scrub, grassland/herbaceous, woody wetlands, and emergent herbaceous wetlands), developed (developed open space, developed low intensity, developed medium intensity, and developed high intensity), agricultural (pasture/hay and cultivated crops), open water, and barren land.

We used the Closest Facility tool in ArcGIS Pro to calculate the fluvial distance between each transect and the nearest dam listed in the CSABI (61). As of September 2022, the CSABI listed 21,047 geolocated dams for Alabama, all of which we included as inputs to the Closest Facility analysis. A disturbance index (64) was calculated from land-use proportions with coefficients ranging from 1 to 10, with higher values indicative of increased disturbance.

2.6 Classification of Hybrids

A Bayesian statistical method was used to assign hybrids between REB and ALB to a hybrid class. The NEWHYBRIDS program computes the posterior probability that a sampled individual belongs to a certain hybrid class based on genotype frequencies (65) from our 64 genotyped SNP loci. There were six hybrid classes assessed in this analysis: (a) pure ALB, (b) pure REB, (c) F_1, (d) F_2, (e) backcrossed individuals from F_1 mating with pure ALB, (f) backcrossed individuals from F_1 mating with pure REB. NEWHYBRIDS only allows for the analysis between two species, so only pure REB, ALB, or their hybrids could be used for this analysis. As such, all other species and multi-species hybrids were removed. The command line version of NEWHYBRIDS was used with a burn-in of 50,000 followed by 100,000 sweeps.

2.7 Statistical Modeling

A generalized linear model was used to test effects of multiple variables on both presence of hybrids and counts of hybrids (Table S4) using the glm function in the statistical software R (66).

3. Results and Discussion

Hybridization is one of the largest threats to native biodiversity in the black basses. Although prior studies demonstrate how widespread introgressive hybridization can occur in formerly allopatric populations of REB and ALB, little is currently known about hybridization between sympatric REB and ALB across a large majority of their native range in the MRB. In 1940, Hubbs and Bailey proposed that hybridization has played an important role in the evolutionary history of both REB and ALB and that ALB might have recently immigrated into the range of REB (24). More recent studies show that REB evolved prior to ALB around three million years ago and are distantly related to ALB (67), but the discordance between nuclear and mitochondrial phylogenies is indicative of a high degree of introgression in the evolution of these species (19). Hybridization between ALB and REB has been shown to occur both in cases where one species is introduced (8, 32) and when both species are introduced (68). There are currently no range-wide estimates of hybridization or information on the role of spatial and temporal reproductive isolation between these species in maintaining species boundaries in systems where they have evolved in sympatry. Characterizing pure and hybridizing populations in these range-restricted black basses is of paramount importance to ensure the conservation of native biodiversity. Here, we surveyed the presence of pure and hybridizing populations of REB and ALB to assess the genetic status of these populations. To our knowledge, this is the first study to utilize a large panel of diagnostic nuclear SNPs to evaluate admixture in a comprehensive sampling of sympatric REB and ALB across much of their native range.

3.1 Sample Collection

In total, 2,679 black bass specimens were collected in this study by electrofishing (N=2,646) and angling (N=33). Fish collected by river system ranged from N=588 in the Cahaba drainage, N=708 in the Tallapoosa River drainage to N=1,383 in the Black Warrior River drainage (Table 1). Fish collected ranged in length from 24 mm to 503 mm TL, with a mean of 214 mm (SD=87) from the Tallapoosa River system, 165 mm (SD = 80) from the Black Warrior River system, and 182 mm (SD = 85 mm) for the Cahaba River system (Table S1). REB were detected at 44 of 58 streams in the Tallapoosa River system, while only found at 15 of 48 streams sampled in the Black Warrior River system, and 16 of 29 sites in the Cahaba River system (Table S2). According to the findings of previous studies (22, 34, 45), we expected to find some evidence of hybridization between REB and ALB due to the geographical overlap between the two species in some stream reaches. Notably, more hybrids were collected than pure ALB in the Black Warrior and Cahaba River systems, and almost as much as pure Redeye Bass in the same systems (Table 1). Detection of hybrids was relatively common throughout streams sampled in all three study systems. The Tallapoosa River system had hybrids present in 24 of 58 sites (41%), while the Black Warrior River and Cahaba River systems had 28 of 48 (58%) and 21 of 29 (72%), respectively (Table S2).

Table 1. Summary of species and hybrids collected from each river system in the Mobile River Basin. Species identity confirmed using 64 diagnostic nuclear single-nucleotide polymorphic loci.

Mobile River Basin Drainage	ALB	REB	LMB	SMB	SPB	Hybrid	N
Tallapoosa	173	363	77	0	0	95	708
Black Warrior	245	542	122	2	0	472	1383
Cahaba	134	230	41	0	0	183	588
	552	1135	240	2	0	750	2679

3.2 SNP Population Genetics Analysis

Formal assignment for ancestry proportions at K=6 of the 2,679 black bass specimens from three different watersheds in the Mobile Basin in Alabama confirmed hybridization among five different black bass taxa, including native ALB, REB, LMB, and non-native Spotted Bass (SPB) and Smallmouth Bass (SMB) (Figure 2; Tables 2 and S2). The majority of genotyped fish collected were REB (N=1,135), followed by hybrids (N=750), and ALB (N=552) with smaller numbers of LMB (N=240) and SMB (N=2) (Table 1). The majority of hybrids collected were between ALB and REB (76%; N=571), followed by hybrids between ALB and SPB (10.5%; N=79), ALB and LMB (2.5%; N=19), and ALB and SMB (N=5). Interestingly, very few LMB X REB hybrids (N=3) or REB X SPB (N=7) hybrids were detected in our study (Table 2). The remaining hybrids (N=65) consisted of various combinations among multiple taxa (Table 2). Hybrids consisting of multiple parental types were more common in the Black Warrior and Cahaba River systems than the Tallapoosa River system indicating there are factors that promote hybridization in those systems (Table 2).

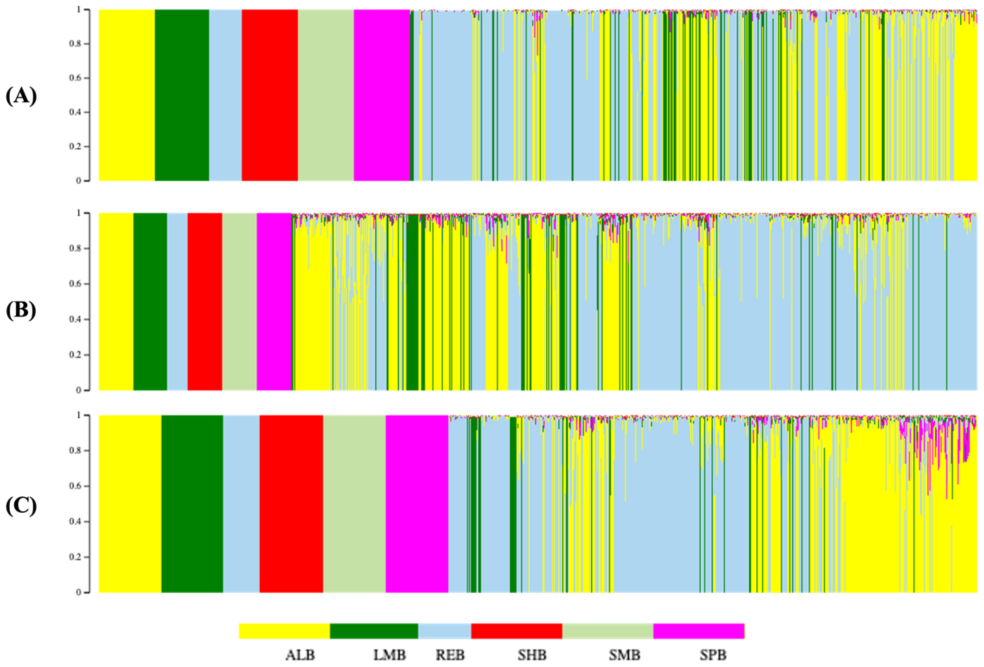

Fig. 2 Taxonomic assignments in STRUCTURE of individuals collected from the (A) Tallapoosa River drainage, (B) Black Warrior River drainage, and (C) Cahaba River drainage. Distinct clusters are illustrated by different colors, with each individual's proportional assignment to those clusters represented by vertical bars. Colors correlate to clusters representing reference species (ALB-Alabama Bass; LMB-Largemouth Bass; REB-Redeye Bass; SHB-Shoal Bass; SMB-Smallmouth Bass; SPB-Spotted Bass).

Table 2. Summary of hybrid types found in each major river system sampled from the Mobile River Basin.

	Number of individuals		
Category	**Tallapoosa River**	**Black Warrior River**	**Cahaba River**
ALB X LMB hybrids	2	12	5
ALB X REB hybrids	80	374	117
ALB X SMB hybrids	0	5	0
ALB X SPB hybrids	9	39	31
LMB X REB hybrids	0	3	0
REB X SPB hybrids	2	3	2
ALB X SMB X SPB hybrids	0	1	3
ALB X REB X SMB hybrids	0	4	0
ALB X REB X SPB hybrids	2	19	14

ALB X LMB X SPB hybrids	0	1	3
ALB X LMB X REB hybrids	0	8	0
LMB X REB X SPB hybrids	0	1	0
ALB X LMB X REB X SPB hybrids	0	2	1
ALB X LMB X SMB X SPB hybrids	0	0	6
ALB X LMB X REB X SMB X SPB hybrids	0	0	0
Total samples	**95**	**472**	**182**

3.3 Tallapoosa River System

The Tallapoosa River system had a low overall hybridization rate of 13%, with more REB (N=363) and ALB (N=173) collected than hybrid individuals (N=95) (Table 1), which was not surprising given that the majority of the Tallapoosa River system is natural and forested with very little habitat disturbance (27). Although admixture was observed in the Tallapoosa River system, it was considerably less than that from the Black Warrior and Cahaba River systems (Figure 2). However, some streams within the Tallapoosa River system, such as the highly developed Sougahatchee creek, had a high rate of hybridization (42%; N=31) (Tables S2) indicating a potential role for stream-level factors responsible for facilitating hybridization. This stream has been affected by the development in nearby cities of Auburn and Opelika, some of the fastest growing cities in Alabama (69). The peak in development led to the clearcutting of forests and effluent from industries combining to increase concentrations of total suspended solids (TSS) entering these Piedmont streams. Piedmont streams in their undisturbed state are naturally characterized by rocky bedrock, boulder, and cobblestone bottoms and are low in sand and silt (70). The equivalent of over 10,000 m^3 of sediment were added per year from nonpoint source pollution sites such as agricultural fields and impervious surfaces that have resulted in a concurrent decline of forest cover and aquatic biodiversity sensitive to pollutants in the

Sougahatchee Creek watershed and likely contributed to a decline in habitat preferred by REB (70) as they typically avoid heavily silted areas (71). The headwaters of Hillabee Creek, another Tallapoosa River tributary, were also heavily logged and have had numerous water-powered mills in its history that may have contributed to the hybridization levels (14-20%; Table S2) observed in that system (72). Other streams may have the influence of agriculture land use within that watershed that has been shown previously to negatively affect the distribution of REB in the Tallapoosa River system (73). These elevated rates of hybridization were relatively rare outside of the main river sites suggesting localized effects have facilitated hybridization between these co-evolved species. Sites along the main river had hybridization rates that ranged from 0-38% (Table S2) and are likely due to the increased interactions of ALB and REB due to habitat overlap in the main river, although they have been shown to utilize different habitat types in the main river (74) lending support for some level of habitat partitioning.

Transect-level observations reveal spatial patterns of hybridization that differ between river systems. REB were commonly detected in the tributaries throughout the Tallapoosa River system, while hybridization was more common at main river sites in the Tallapoosa River system (Figure 3). We would expect hybridization to decrease with upstream sampling; however, there is no consistent pattern observed in our data. Hybridization was detected throughout the stream reaches sampled or in some cases, only in the higher stream reaches relative to the lower sites (Figure 3), illustrating the movement of hybrids into the uppermost reaches of these streams. There were nine sites that had hybridization rates that exceeded 10% with sample sizes over 10 individuals, and five of those were main river sites (Figure 3 and Table S2). The other four streams are distributed throughout the Tallapoosa River watershed and are High Pine Creek

(38%), Sougahatchee Creek (42%), Uphapee Creek (11%), and Wedowee Creek (20%) indicating that hybridization is occurring in select streams across the Tallapoosa River watershed (Table S2). Interestingly, although REB X ALB hybrids are the most commonly hybrid type detected, parental types of both species were detected in the same sample sites in several streams from the Tallapoosa River system without the presence of any hybrids (Figure 3). This could provide support for localized effects facilitating hybridization or simply a lack of detection of hybrids in those streams. REB also co-occur with Largemouth bass at many sites with hybrids between the two only accounting for 2% of the overall observed hybridization (Figure 3 and Table 2) likely due to habitat partitioning between these two ecologically different species.

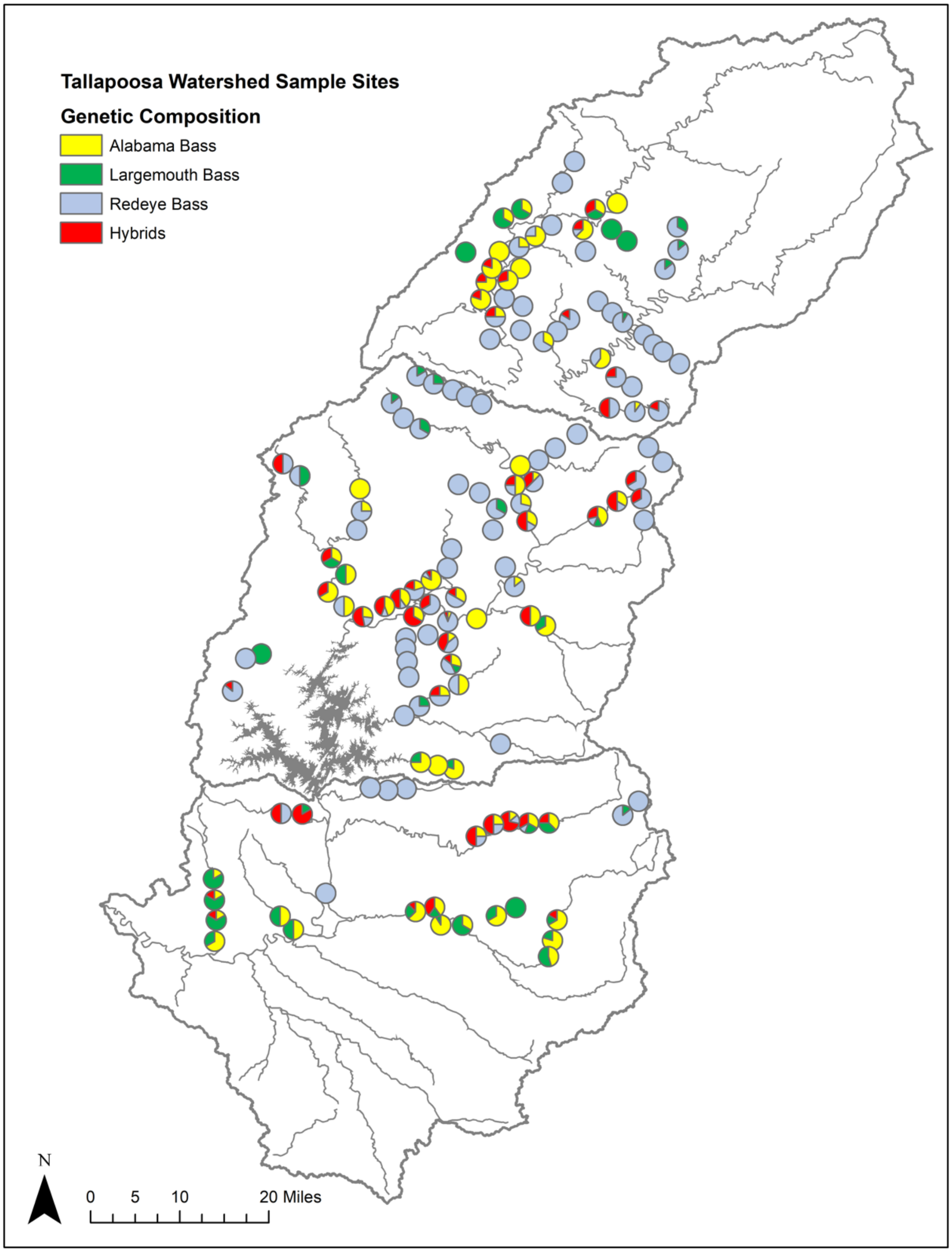

Fig. 3 Collection sites for black bass specimens from the Tallapoosa River system, genotyped using 64 single-nucleotide polymorphism loci that are diagnostic for six *Micropterus* spp. Pie charts illustrate the number of pure or hybrid individuals collected at each site.

3.4 Black Warrior River System

The Black Warrior River system had an overall hybridization rate of 34% with almost as many hybrids collected (N=472) as REB (N=542) and almost two times as many ALB (N=245) (Table 1). These results reveal substantial hybridization with more hybrid combinations and only a few populations of REB present, most of which occur in the Bankhead National Forest (Figure 4). Only two streams (Hubbard Creek and Tedford Creek) containing REB had no hybrids detected, while the remaining streams had hybridization rates ranging from 4-59% (Table S2). Multiple streams that had historical populations of REB (Brushy Creek, Broglan Creek, Calvert Prong, Gurley Creek, Little Warrior Creek, Lost Creek, Ryan Creek, Slab Creek, and Valley Creek) are now composed of ALB X REB hybrids or ALB with zero detections of REB (Table S2) indicating an extirpation of REB from those systems (22, 75).

A previous study suggested a distributional decline of REB in the Black Warrior River drainage of the MRB and recommended a survey to inform the establishment of conservation measures (22). A later survey by the US Forest Service (USFS) indicated that REB had been replaced by ALB in the impounded reaches and lower to middle transitional zones of Brushy Creek indicating an increase in the range of ALB and a concomitant decline in occupancy of REB in their native range as a result of habitat alteration by the construction of Lewis Smith Reservoir (75). Other studies have also shown that the prevalence of ALB increases following impoundment (76, 77). Our sampling confirmed the negative trend by only detecting four *Micropterus* spp., two hybrids between REB X ALB and two pure Largemouth Bass from Brushy Creek (Table S2). These data show that ALB ranges are extending into stream reaches where REB historically are the dominant black bass species, resulting in hybridization with REB. Another historical REB stream and site of the holotype (22) for that species description, Calvert

Prong had a hybridization rate of 74% (N=68) with zero detections of REB (Table S2). REB were not detected in the Mulberry Fork or Upper Black Warrior streams which are proximal to the Warrior Coal Field which has a history of intensive mining dating back to at least the 1800's (78). Due to lack of historical data, it is not known if REB inhabited those streams at one time. The habitat types related to REB occurrence are present in those systems (26), so it is possible that mining has extirpated those populations. Some components of coal and coal mining waste have been shown to be toxic to fish at low concentrations (79).

Only one stream system, Five Mile Creek, showed some trend of longitudinal hybridization with relatively more genetically pure REB in the upper reaches, while hybrids and ALB predominate the lower reaches (Figure 4). Five Mile Creek, one of the most polluted waterways in Alabama (80, 81), had a hybridization rate of 55% (N=215; Table S2). This creek has been shown to have a lack of species sensitive to disturbance relative to other streams in the Black Warrior River drainage due to the headwaters of this stream being heavily developed, with good habitat in the middle, and the lower reaches being more residential (80). The trend of fragmented habitat and ecological impacts is reflected in our data with hybridization occurring at the uppermost sample site and in the lower sample sites, while the middle sites are absent of hybrids (Figure 4). There are documented cases of industrial pollution from coal mining and other industries that combine with urban runoff to create water that is of poor quality and dominated by only a few tolerant species indicating species diversity is impacted by human disturbance (80, 81). Another study found similar results due to a dolomite mine quarry discharge resulting in reduced water quality and a shift in more pollution- and sediment-tolerant macroinvertebrates (81). Blackburn Fork also had a high hybridization rate (52%) and was similarly determined to have lower habitat scores due to agricultural runoff, residential development, and impacts due to damming to create Inland Lake (80). In addition to the above, there are numerous concentrated animal feeding operations (CAFOs) located in the eastern portion of the Black Warrior River system (unpublished data, Black Warrior Riverkeeper) that correlates with the high rates of hybridization in that area. The literature is full of examples of how CAFOs operations have had deleterious impacts on the water quality of adjacent waterways, specifically nutrient pollution that has resulted in numerous major fish kills (82, 83).

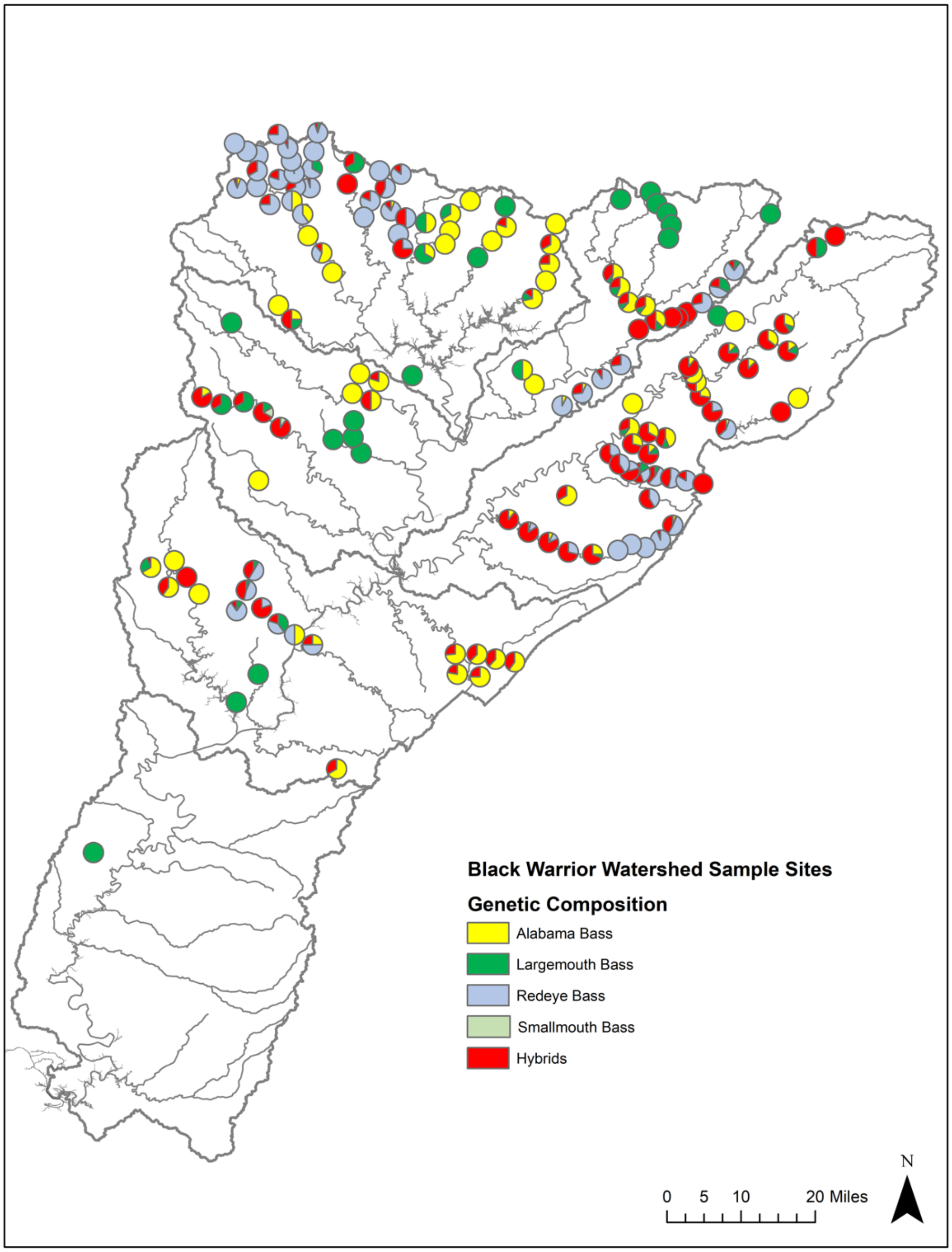

Fig. 4 Collection sites for black bass specimens from the Black Warrior River system, genotyped using 64 single-nucleotide polymorphism loci that are diagnostic for six *Micropterus* spp. Pie charts illustrate the number of pure or hybrid individuals collected at each site.

3.5 Cahaba River System

The Cahaba River system had more pure populations of REB than the Black Warrior River system, but less than the Tallapoosa River system. A similar situation was observed in the Cahaba River system had an overall hybridization rate of 31% and almost as many hybrids (N=183) were collected as REB (N=230) and more than ALB (N=134) (Table 1). The Cahaba River system had more pure populations of REB than the Black Warrior River system, but less than the Tallapoosa River system. However, there were streams with high rates of hybridization (56-75%) that mirrored the results observed in some of the Black Warrior River streams (Table S2). These streams were located throughout the upper and middle portion of that river system (Figure 5) indicating hybridization is variable and more localized to certain stream systems. A small number of ALB and REB were detected in the same reach of one stream with no hybrids detected (Figure 5). All of the main river sites sampled contained hybrids with hybridization frequency ranging from 7-75% at those sites (Figure 5; Table S2). Only the upper Cahaba River site in Trussville had a lower hybridization rate (13%) with a substantial number (N=82) of REB collected with zero ALB (Table S2). Moving southward, hybridization levels at the main river sites increased substantially (31-75%) before decreasing once again outside the range of REB (7%) then rising again (41%) at the lowermost site (Figure 5; Table S2), indicating the potential of hybrids to disperse from source populations.

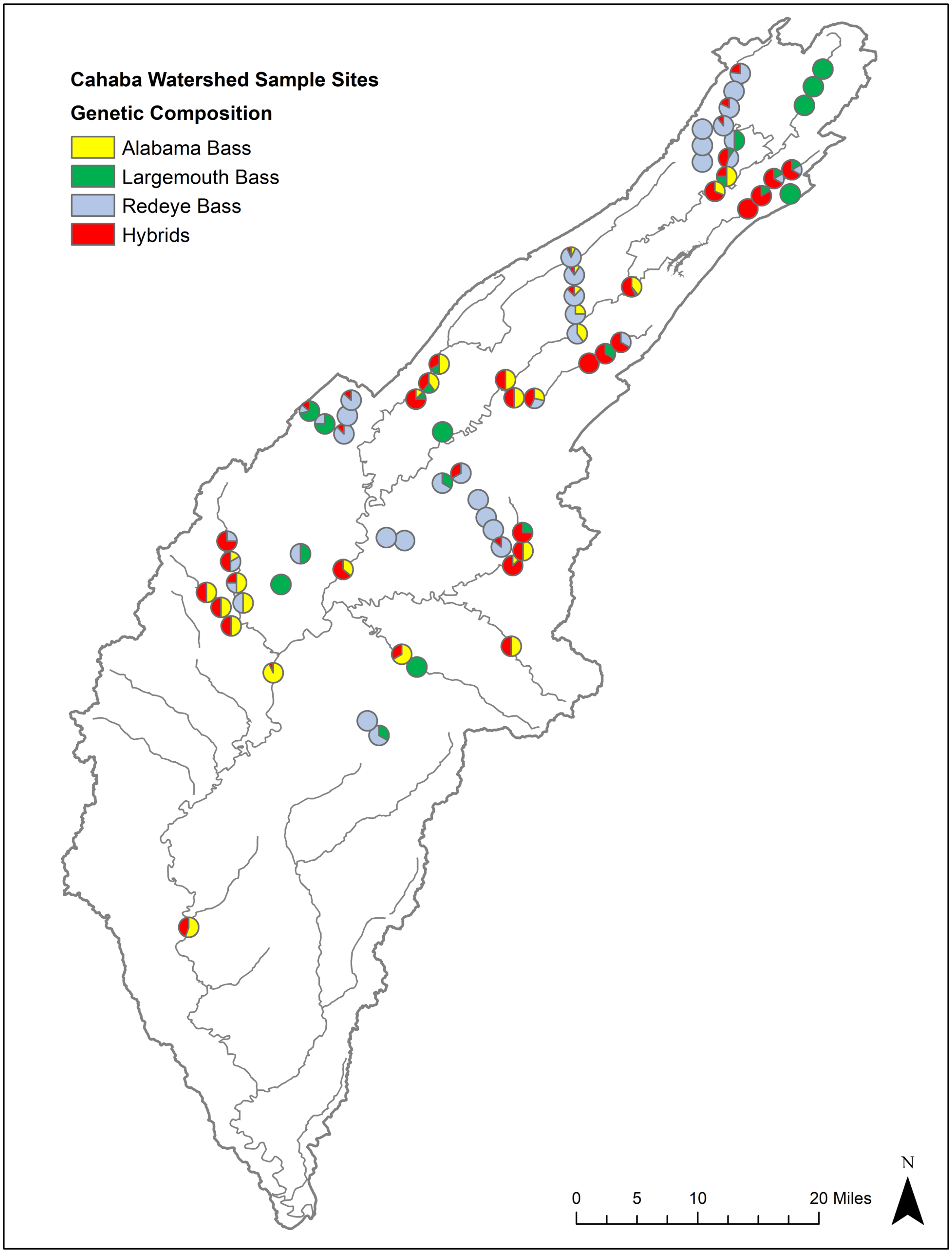
Cahaba Watershed Sample Sites
Genetic Composition
Alabama Bass
Largemouth Bass
Redeye Bass
Hybrids
N
0
5
10
20 Miles

Fig. 5 Collection sites for black bass specimens from the Cahaba River system, genotyped using 64 single-nucleotide polymorphism loci that are diagnostic for six *Micropterus* spp. Pie charts illustrate the number of pure or hybrid individuals collected at each site.

The metropolitan influence of Birmingham, Alabama's largest city, has been shown to reduce the abundance of disturbance-sensitive taxa and increase the abundance of disturbance-tolerant taxa in response to pollution and sedimentation in the Cahaba River (84). Despite being one of the most biologically diverse rivers in the United States (48, 85, 86), the Cahaba River system has experienced multiple extirpations and a general decline in taxonomic diversity as ichthyofaunal assemblages shift to more disturbance-tolerant taxa due to impairment by increased sedimentation (71, 87). These changes in the last few decades have occurred concurrently with widespread urbanization and resulting decline in habitat and water quality (87, 88). Our data suggest that REB are not immune to the detrimental effects of anthropogenic disturbance. Notably, Shoal Creek had a hybridization rate of 75% (N=16; Table S2) and was the site of the holotype of REB from the Cahaba River system (22). Our sampling did not detect any pure REB in Shoal Creek and unfortunately detected more hybrids than all other species combined (Table S2). High concentrations of many pollutants have been frequently found in the uppermost reaches of the Cahaba River (87, 89), with suspended solids being the largest water pollutant in the Upper Cahaba River system.

Agricultural land use can elevate turbid conditions in lotic systems that promote interspecific interactions. The presence of hybrids between naturally sympatric Red Shiners (*Cyprinella lutrensis*) and Blacktail Shiners (*C. venusta*) has been linked to turbid conditions resulting from excessive sedimentation in the Guadalupe and San Marcos rivers of Texas (90), with abatement of hybridization in these systems as water clarity improved (91). Increased turbidity can affect prezygotic isolating mechanisms by eliminating visual recognition and assortative mating cues (92-94). The scarlet-colored operculum fringe of Redear Sunfish (*Lepomis microlophus*) has been shown to play a role in species recognition and preventing hybridization with Bluegill (*Lepomis microchirus*). Once removed, by physical removal and obscurement due to turbid conditions, the two species readily hybridized (92). These experiments illustrate the importance of color and visual recognition as a reproductive isolating mechanism in the sunfishes and indicates a potential role for how habitat alteration and other anthropogenic factors could relax sexual selection between sympatric populations of ALB and REB. The concealment of visual cues such as the blue eye crescent and white caudal fin margins of redeye bass could lead to a breakdown of reproductive isolating mechanisms in these co-occurring species by challenging the female's recognition of conspecific males. There have been very high levels of turbidity measured on the Little Cahaba due to high rains and associated runoff from adjacent construction sites, a factor that could explain the high levels of hybridization (74%) observed at that site (89). In addition, the Little Cahaba River is impounded to form Lake Purdy, one of the primary water sources for metropolitan Birmingham. As such, it is frequently affected by Birmingham Water Works Board water withdrawals that reduce the amount of water available (87, 89) and elevated nutrient levels in wastewater discharge that promote low dissolved oxygen which may negatively affect REB populations (88). Similarly, Shades Creek also had a high rate of hybridization (53%) and is considered an impaired stream to support fish and wildlife due to sedimentation loadings from non-point pollution sources with poor index of biotic integrity (IBI) scores ranging from poor in the upper watershed to fair in the lower watershed relative to other reference sites in the Cahaba River watershed (95, 96). Notably, these subpar biological assessment scores coincided with high levels of turbidity (95). Schultz Creek also had a high rate of hybridization (38%) and might be due to historical impacts of mining as it is a part of the Cahaba coal field (97).

3.6 Statistical Modeling Analyses

As expected, hybrid count detection increased with higher coefficients of disturbance index. For each unit increase in disturbance index coefficient, we detected 3.87 (P=2.62e-13; 2.67-5.59 95% CI) times as many hybrids as indicated by the generalized linear model regression. The removal of habitat-specific niches can enhance the survival of hybrids which can exacerbate the effects of hybridization (16, 98). Streams impacted by anthropogenic disturbances have been shown to shift fish assemblages to more tolerant and generalist fishes while streams surrounded by heavily forested land cover has a more diverse fish assemblage composed of more specialists (40). Relative abundance of hybrids has been positively correlated with anthropogenic habitat disturbances such as increased agricultural land use (35, 98) while occurrence of pure REB have been associated with more forested watersheds in Piedmont streams with their occurrence affected by levels of development (59). Another study showed that REB were detected in areas with lower levels of disturbance at both the watershed and local scale (99) indicating sensitivity to habitat changes. A more recent study found that the highest numbers of REB from the Black Warrior system were detected in the heavily forested Bankhead National Forest with their occurrence lower in areas with a higher disturbance index (26). The majority of REB populations occurred in areas with low levels of disturbance indicates that habitat disturbance negatively affects REB. Our data reflect that trend with genetically pure populations located in areas with little disturbance.

The lower numbers of genetically pure REB in their native range of the Black Warrior and Cahaba River systems coupled with the prevalence of hybrids indicates that hybridization with sympatric Alabama Bass is negatively affecting REB populations. Notably, the observed hybridization rates in many streams equals or exceeds those from populations where hybridization results from an introduction of one species into the native range of another (12, 14, 15, 55, 98). For example, hybridization rates between native Guadalupe Bass (*M. treculi*) in Texas with introduced SMB were 46% or less (100), after a minimum of a couple of decades of hybridization (101). These numbers are almost half of the highest rate (75%) that we observed in our study, and less than many other of our sampled streams. Not only was the frequency of hybridization higher in the Black Warrior and Cahaba River systems, but there was a greater number and variety of multi-species hybrid combinations detected. SPB alleles were found in all three study systems, but the Black Warrior and Cahaba River systems also had nonnative SMB alleles, with evidence of all non-native species hybridizing with native fish. These nonnative introductions could work in concert with habitat disturbance to amplify hybridization in these two systems. The Black Warrior and Cahaba River systems are both located near the largest city in Alabama (Birmingham) and are therefore associated with urbanization and historical and current mining operations.

Habitat fragmentation from damming rivers can create a transition zone characterized by the merging of lentic and lotic systems that can facilitate interspecific interactions and hybridization by removing reproductive isolation between species (102). All three study river systems contain large lock and dam structures with additional impoundments on many tributaries. The Black Warrior River system alone has almost 4,000 dams and the Cahaba River system has close to 1,000 dams, compared to less than 100 in the Tallapoosa River system (unpublished data, Southeast Aquatic Resource Partnership), which could lead to altered habitat that favors ALB and ALB X REB hybrids. Hybrids may be able to better navigate these transitional habitats created by disturbance due to a more diverse assemblage of genotypes allowing them to maintain source populations while migrating to other areas, perpetuating and exacerbating the hybridization with REB in the MRB (103). Hybrid individuals have been shown to migrate from source populations to preferentially establish their genomes in new habitats (16, 98). Hybrids between ALB and REB in the Upper Savannah River Basin were shown to migrate to lakes previously free of ALB and preferentially mate with REB, creating a hybrid swarm (20). Hybrids between invasive red shiners and native black shiners in the upper Coosa River system dispersed via river mainstems and rapidly colonized tributaries with an affinity for tributaries with native congeners. First filial and later generation hybrids between Rainbow Trout (*Oncorhybchus mykiss*) and Cutthroat Trout (*O. clarkii lewisi*) have been shown to disperse over large distances (> 50 km) to introduce their non-native alleles to native Cutthroat Trout populations (104). Our data show that for each one-kilometer increase in distance to nearest dam, we encountered a 1.9% (P=0.05; 0.04-3.7 95% CI) decrease in the odds of encountering hybrids, indicating the habitat disturbance created by dams are correlated with increased numbers of hybrid individuals. Elevation also seemed to be a limiting factor for hybrid numbers, which has been shown previously to increase the odds of REB occurrence with increasing elevation (59). For each 100-meter increase in elevation, we detected a 14.5% (P=0.04; 0.04%-3.7% 95% CI) decrease in the number of hybrids in our study systems.

3.7 Classification of Hybrids and Hybrid Zone

Hybrid zone structure is an important consideration when investigating hybridization dynamics between species with geographical overlap. Hybrid zones are created when hybrid offspring survive and pass alleles to future generations (10). The suite of diagnostic markers used in this study are sensitive to even low levels of hybridization allowing us to detect low levels of admixture. The majority of hybrids captured in this study were later-generation or back-crossed individuals with very few F1 hybrids indicating that interactions between REB and ALB are infrequent. The increased number of hybrids backcrossed to ALB in the Black Warrior River system (Table S3) is interesting since the number of REB are more abundant than ALB in that system (Table 1). The predominance of hybrid individuals in the Black Warrior and Cahaba rivers suggest a panmictic hybrid swarm dominated by later generation hybrids and characterized by a complete breakdown of reproductive isolating mechanisms (16, 105). The presence of hybrid swarms are typically observed when formerly allopatric populations of black bass are brought together by translocations (7, 13, 14, 105); however, habitat disturbances can have similar effects as nonnative introductions in relaxing reproductive isolating mechanisms (93). Anthropogenic activities on landscapes and riverscapes continue to reduce species richness and genetic diversity by agriculture (106, 107), reservoir construction (108, 109), and urbanization (110, 111). It is possible that habitat disturbance in these systems has facilitated more interactions between ALB and REB in the MRB than would otherwise be possible in an un-disturbed setting.

Run independently from STRUCTURE, the NEWHYBRIDS analysis revealed similar patterns of admixture observed in the STRUCTURE bar plots (Figure 6) and confirmed that most hybrids present in all three basins sampled are backcrossed individuals (Table 3). The direction of backcrossing differs between river systems with a higher frequency of hybrids backcrossing occurring with ALB in the Black Warrior River system, backcrossing with REB in the Cahaba River system, and backcrossing equally with both ALB and REB in the Tallapoosa River system (Table 3). There were very few F1 individuals in all three river systems relative to other hybrid classes (Table 3). The scarcity of F1 individuals coupled with the predominance of backcrossed individuals suggests a bimodal hybrid zone (105). These populations of ALB and REB might exhibit strong assortative mating typically as a prezygotic barrier to gene flow, but have a breakdown in postzygotic selection due to genomic incompatibilities or failure to adapt to environmental conditions (105).

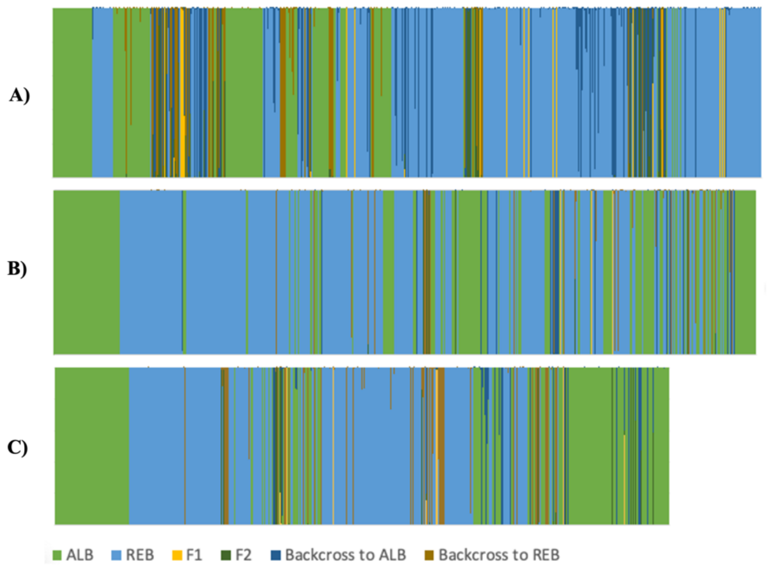

Fig. 6 Results from the NEWHYBRIDS analysis with each line representing an individual's posterior probability to a hybrid class.

Table 3. Summary of hybrid classes between ALB and REB as identified by NEWHYBRIDS.

Class	Warrior	Tallapoosa	Cahaba	Total
ALB	303	188	219	710
REB	644	376	248	1268

F1	29	3	6	38
F2	46	5	10	61
F2 backcross X ALB	86	22	14	122
F2 backcross X REB	54	22	41	117

When compared to STRUCTURE results, there was 91% agreement between programs in identifying pure and hybrid individuals (Table S3). However, there were some differences in pure and hybrid classification between the two programs. There were 87 individuals identified as hybrids with STRUCTURE that were assigned as pure ALB with NEWHYBRIDS. Similarly, NEWHYBRIDS identified 135 pure REB that were classified as hybrids with STRUCTURE. This discordance is likely due to the highly conservative Q-value threshold of ≥0.95 for species purity in the STRUCTURE results as most of the discrepant individuals had Q-values ranging from 0.800 to 0.949.

4. Conclusion

Southeastern fishes are highly endemic and imperiled (112, 113) and Alabama has already experienced an extirpation of Shoal Bass (*M. cataractae*) from its waters due to habitat loss and lack of monitoring . The limited range of REB makes them vulnerable to loss of genetic diversity due to hybridization with sympatric ALB. Given the high detections and genetic purity of REB in Bankhead National Forest relative to the rest of the Black Warrior River system, conservation efforts should be focused on protecting these populations. The data generated here should augment future studies as there are still no published works on the basic life history characteristics such as diet, spawning, and age and growth.

This study provides a baseline for future investigations into clinal and spatial components of hybridization and demonstrates that genetic assessments are crucial to managing these populations by identifying the presence of pure and hybridizing populations. The use of genetics to quantify and qualify species distributions delineated by the different drainages studied here adds valuable data to assessing the species status of these newly described bass. Our data should reflect a conservative estimate of the hybridization occurring between REB and ALB in the MRB as all possible streams were not sampled and not every section of each stream was able to be sampled. Further research is needed to better elucidate the abiotic and anthropogenic factors that may facilitate gene exchange between these co-evolved species to better manage and conserve the remaining pure populations of REB in the MRB. Future studies will aim to expand the surveys to the Coosa River system and continue to develop ecological modeling utilizing the hybridization data to develop mitigation plans in effort to ameliorate current hybridization and prevent future hybridization in genetically pure populations of REB.

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